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Micropropagation, phenotyping and genotyping of chestnut progenies obtained from controlled crosses

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Resumo

Phytophthora cinnamomi é um oomiceta que infecta as raízes e pode causar a morte de árvores pertencentes à família Fagaceae, como as do género *Castanea*. Atualmente a doença da tinta é considerada a maior ameaça na Europa para os castanheiros, originando uma acentuada diminuição na produção de castanhas, que constitui o maior rendimento das regiões montanhosas de Trás-os-Montes. Assim, o melhoramento genético para a resistência é muito importante para a economia nacional, visando o desenvolvimento de novas variedades de castanheiro, menos suscetíveis a esse patógeno.

A espécie Europeia, *Castanea sativa* Mill., é suscetível à *P. cinnamomi*, enquanto que as espécies Asiáticas são resistentes devido à sua coevolução com o patogénio. O INIAV iniciou um programa de melhoramento baseado em cruzamentos controlados, utilizando espécies Asiáticas, *Castanea crenata* Sieb. & Zucc. (Japonesa) e *Castanea mollissima* Blume (chinesa) como doadoras de resistência e a espécie Europeia, sensível, como progenitora feminina. O objetivo destes cruzamentos controlados foi obter descendências com resistência melhorada à *P. cinnamomi*.

Durante esta tese, foi realizada a fenotipagem e genotipagem da descendência de cruzamentos controlados com a finalidade de selecionar os indivíduos mais resistentes. A associação de padrões de análise de fragmentos com dados de fenotipagem podem permitir a seleção de genes potencialmente associados à resistência. A genotipagem foi realizada com 16 microssatélites em 47 genótipos e o resultado foram 4 alelos com possível relação com a resistência. Também foi realizada a micropropagação dos genótipos SM904, SC55, SC1202 e SC914, já selecionados com resistência melhorada durante o projeto em curso, onde se encaixa a presente tese. Foram feitos cálculos para taxa de multiplicação e percentagem de enraizamento, importantes para a caracterização dos genótipos. O genótipo SM904 apresentou o maior resultado para ambas as análises.

Este trabalho contribui para um dos objetivos do atual programa de pesquisa, que consiste na obtenção de novos genótipos com resistência melhorada à *P. cinnamomi* para serem utilizados como porta-enxertos compatíveis para as variedades nacionais de castanhas.

Palavras-chave: Infeção, agente patogénico, seleção, micropropagação, microssatélites.

Abstract

Phytophthora cinnamomi is an oomycete that infects the roots and can cause the death of trees belonging to the Fagaceae family, such as genus *Castanea*. Ink disease is currently considered the major threat to chestnut trees in Europe, causing a high decrease in nut production, which is the highest income of mountain regions of Trás-os-Montes. Thus, genetic improvement for resistance aiming to developing new chestnut varieties less susceptible to this pathogen is very important for the national economy.

The European species, *Castanea sativa* Mill., is susceptible to *Phytophthora cinnamomi*, while Asian species are resistant due to coevolution with the pathogen. INIAV initiated in 2006 a breeding program based on controlled crosses, using Asian species, namely *Castanea crenata* Sieb. & Zucc. (Japanese) and *Castanea mollissima* Blume (Chinese) as donors of resistance, and the sensitive European species as female progenitor with the objective of obtaining segregating progenies for the resistance character.

During this thesis, phenotyping and genotyping of progenies of crosses performed in 2016 was carried out in order to select the most resistant individuals and try to associate resistance with fragment analysis patterns. This phenotyping-genotyping analysis was performed with 16 microsatellites in 47 genotypes and the result was 4 alleles that may be related to resistance. Was also performed the micropropagation of the genotypes SM904, SC55, SC1202 and SC914, already selected with improved resistance during the project on course, where this thesis fits. Calculations for multiplication rate and rooting percentage were made, important for the characterization of the genotypes. Genotype SM904 showed the highest result for both analysis.

My work also contributes to one of the objectives of the current research program, which is to obtain new genotypes with improved resistance to *Phytophthora cinnamomi* to be used as rootstocks compatible for grafting with the national chestnut varieties of nut production.

Key words: Infection, pathogenic agent, selection, micropropagation, microsatellites.

Resumo alargado

Phytophthora cinnamomi é um oomycete que infeta as raízes e pode causar a morte dos castanheiros, do género *Castanea*, bem como as plantas de outros géneros pertencentes à família Fagaceae, como *Quercus*. A podridão radicular provocada por este patogénio dá origem a um défice de absorção de água e nutrientes, o que pode resultar na morte da árvore. Esta doença é considerada atualmente a maior ameaça para a produção de castanha, que constitui o maior rendimento da região de Trás-os-Montes. Assim, o melhoramento genético para a resistência é importante para a economia nacional, com o objetivo de desenvolver novos genótipos menos suscetíveis a este patogénio.

A espécie Europeia, *Castanea sativa* Mill., é suscetível à *Phytophthora cinnamomi*, por outro lado as espécies Asiáticas apresentam resistência devido à coevolução com o patogénio. O INIAV iniciou em 2006 um programa de melhoramento genético baseado em cruzamentos controlados, utilizando as espécies Asiáticas, nomeadamente *Castanea crenata* Sieb. & Zucc. (Japonesa) e *Castanea mollissima* Blume (Chinesa) como polinizadores e a espécie Europeia, sensível, como progenitor feminino, com o objetivo de obter descendências segregantes para o caracter resistência. Apesar de as espécies Asiáticas serem resistentes ao patogénio, possuem castanhas de inferior qualidade e têm baixa compatibilidade de enxertia com as variedades Europeias. Como em Portugal a cultura de castanheiro tem como principal objetivo a produção de castanha, as variedades Asiáticas não são uma alternativa viável.

Durante esta tese foram micropropagados 4 genótipos anteriormente selecionados, pela resistência melhorada à *P. cinnamomi* e foi realizada a fenotipagem e genotipagem de híbridos de cruzamentos realizados em 2016, em que o progenitor feminino é a variedade Bária da espécie *C. sativa* e o progenitor masculino é a espécie *C. crenata*, dador de resistência. Para o cruzamento controlado foi usada uma mistura de pólen de duas árvores de *C. crenata*, de forma a aumentar o sucesso de fertilização. O objetivo da fenotipagem e genotipagem foi selecionar os indivíduos com carácter resistente e tentar associar a resistência com padrões de análise de fragmentos obtidos por amplificação em PCR de microssatélites.

Foi calculada a taxa de multiplicação e percentagem de enraizamento para os genótipos SM904, SC55, SC1202 e SC914, anteriormente selecionados. Estes dados são importantes para a caracterização dos genótipos que fazem parte do projeto em curso, onde se encaixa a presente tese. A técnica de micropropagação permite a rápida

multiplicação de material vegetal em condições estéreis com temperatura e fotoperíodo controlados. O padrão da taxa de multiplicação e percentagem de enraizamento foi o mesmo: o genótipo SM904 obteve os resultados mais elevados, com taxa de multiplicação de 2.54 e 79.6% de enraizamento. De seguida o genótipo SC55, seguido do SC1202 e por último o genótipo SC914, com os valores mais baixos. Segundo Santos *et al*. (2015) os genótipos SM904 e SC55 são os mais resistentes entre estes quatro, sendo também os que melhor multiplicam e enraízam. Este resultado poderá indicar uma relação entre resistência e vigor.

A fenotipagem consistiu na análise dos sintomas das plantas após inoculação com o agente patogénico. Esta inoculação consistiu na mistura de *P. cinnamomi* com o solo em que as plantas se encontravam. Os sintomas foram anotados durante os 100 dias de ensaio. No último dia do ensaio as plantas foram retiradas do solo e as suas raízes foram verificadas, tendo sido preenchida uma tabela com a percentagem de podridão radicular, comprimento de lesão e foi dada uma nota para o nível de suscetibilidade, segundo Santos *et al*. (2015). As plantas vivas foram então enraizadas de novo, nas mesmas condições. Comparando os resultados obtidos nesta tese com os obtidos por Santos *et al*. (2015) é possível concluir que 100 dias são insuficientes para fenotipar plantas com quatro anos. Enquanto em Santos *et al*. (2015) apenas 14% de plantas com seis meses sobreviveram, neste trabalho obteve-se 94% de sobrevivência em plantas com quatro anos, o que poderá indicar que em plantas maiores/mais velhas terão maior resistência/capacidade de regenerar o sistema radicular, e portanto é necessário mais tempo para que ocorra a infeção e a proliferação do patogénio, sendo sugerida nesta tese o aumento para 1 ano do tempo de inoculação. Foram utilizadas 113 plantas da descendência do cruzamento de *C. sativa* (variedade Bária e Colarinha) e *C. crenata*, nomeadamente descendências BAC, BCC, BDC e CFC. Plantas de *C. sativa* e *C. crenata* foram usadas como controlo.

A genotipagem foi feita com 16 microssatélites para 47 genótipos do cruzamento BDC (*C. sativa* variedade Bária x *C. crenata*). Folhas destes genótipos foram colhidas na Primavera e a extração de *DNA* foi realizada com protocolo *CTAB*. Foram utilizados *Qubit* e *Nanodrop* para verificar a concentração e qualidade de *DNA*, seguindo-se a purificação e diluição das amostras de DNA. Foram feitos *PCRs* com os 16 pares de *primers* utilizados, seis “Cc” (desenhados em transcritomas de *C. crenata*) e dez “Cm” (desenhados em transcritomas de *C. mollissima*), por Santos *et al*. (2015) e Kubisiak *et al*. (2013), respetivamente. Através das ferramentas *Primer-BLAST* e *UniProt*, os genes

amplificados por estes *primers* foram estudados, bem como a sua função e regulação prevista.

A resistência é uma característica quantitativa, envolvendo, portanto, uma grande quantidade de genes. Foram usados microssatélites para a genotipagem por estes terem grande capacidade de deteção de polimorfismos, conseguindo assim distinguir indivíduos diretamente relacionados (indivíduos de uma mesma descendência). Estes marcadores moleculares são utilizados para testes de paternidade e análises de *Quantitative Trait Loci*, porções do genoma ligadas a uma determinada característica quantitativa de interesse. O facto de as características quantitativas terem grande influência ambiental torna difícil a seleção desta característica pela relação indireta de fenótipo-genótipo. A associação dos dados de fenotipagem e genotipagem permitiram selecionar 4 alelos possivelmente interessantes para a identificação dos indivíduos resistentes à *P. cinnamomi*, sendo um acrescento à informação pré-existente. Os alelos 141 e 145 (primer Cm735), 177 (primer Cm433) e 272 (primer Cm361) estão presentes apenas nos indivíduos que, durante o processo de fenotipagem, não demonstraram qualquer sintoma resultante da infeção pelo patogénio ou demonstraram apenas podridão radicular. Os genótipos foram agregados em 5 diferentes grupos: sem sintomas (“nosympt”), níveis 2 e 3 de podridão radicular (“rootrot23”), níveis 4 e 6 de podridão radicular (“rootort46”), lesão externa do colo (“lesion”) e as plantas que morreram devido à infeção com *P. cinnamomi* (“dead”). As frequências alélicas obtidas em cada grupo foram calculadas com o software PopTree e o resultado apresentado numa árvore *Neighbor-Joining*, que uniu os grupos “sem sintomas” e “níveis 2 e 3 de podridão radicular”. Os grupos “lesão” e “mortos” ficaram agrupados, o que indica que existem mais alelos em comum entre os indivíduos com o máximo de suscetibilidade (mortos) e os que apresentaram lesão externa do colo do que com os que apresentaram podridão radicular. Este resultado poderá indicar de que o sintoma lesão é mais grave e, portanto, mais ligado a suscetibilidade do que a podridão radicular. Sendo que a lesão do colo é o resultado da passagem do patogénio das raízes para os feixes vasculares, faz sentido este ser o sintoma mais diretamente ligado à suscetibilidade. Desta forma, foi feita a seleção dos alelos presentes apenas nos indivíduos sem sintomas ou com podridão radicular, pois estes alelos poderão estar envolvidos com algum tipo de resistência ao avanço do patogénio. Os alelos comuns entre os indivíduos que, durante o processo de fenotipagem, apresentaram lesão do colo ou morreram, estarão muito provavelmente envolvidos com a suscetibilidade.

Através das frequências alélicas obtidas para cada genótipo foi possível identificar o progenitor masculino para alguns genótipo, uma vez que a polinização foi realizada com mistura de polén de 2 indivíduos, bem como determinar a percentagem de heterozigotia em cada *locus*. O *locus* que apresentou maior heterozigotia foi o Cm559, com 93.6% dos genótipos heterozigóticos para este *locus*. Apenas 8 *loci* dos 16 permitiram a identificação parental em alguns genótipos, sendo que o Cc30 foi aquele que apresentou maior percentagem de genótipos com progenitor masculino identificado, cerca de 57%.

A identificação de alelos ligados à resistência para a *P. cinnamomi* possibilitará a seleção de forma mais expedita de genótipos de interesse, que apresentem resistência à *P. cinnamomi*. Este trabalho contribui também para alcançar um dos objetivos do programa de investigação em curso no INIAV, que consiste na seleção e propagação de novos porta-enxertos com resistência melhorada à *Phytophthora cinnamomi* e compatíveis para enxertia com as variedades nacionais de castanha.

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Abbreviations

µL – microliter

µM - micromolar

ABA – Abscisic Acid

AC – Activated Charcoal

BAC – Bária (tree A) x *C. crenata*

BCC – Bária (tree C) x *C. crenata*

BDC – Bária (tree D) x *C. crenata*

bp – base pair

C1 – *C. crenata "Monte"*

C2 – *C. crenata "Casa"*

CFC – Colarinha (tree F) x *C. crenata*

cm – centimeter

CTAB – Cetyl Trimethylammonium Bromide

DNA – Deoxyribonucleic Acid

EEO – Electroendosmosis

EST-SSRs – Simple Sequence Repeats derived from Expressed Sequence Tags

Ho – Heterozygosity observed

HR – Hypersensitive response

HPDP – Hybrids whose Paternity is Distinguished by a given Primer

IBA – Indolbutyric Acid

L – liter

MAP – Mitogen-Activated Protein

mL – milliliter

MPK – Mitogen-Activated Protein Kinase

MSB – Murashige & Skoog + Benzilaminopurine

mM - millimolar

NJ (Neighbor-Joining)

ng – nanogram

nm – nanometer

OWB – Organell Wash Buffer

PAMPs – Pathogen-Associated Molecular Patterns

PAT – Topoisomerase 2-Associated Protein

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

PIN – Peptidylprolyl Cis/Trans Isomerase NIMA-Interacting

PRRs – Pattern Recognition Receptor

rpm – revolutions per minute

SA – Salicylic Acid

SC – *C. sativa* x *C. crenata*

SM – *C. sativa* x *C. mollissima*

SSRs – Simple Sequence Repeats

TBE – Tris-borate-EDTA

VLCFA – Very-Long-Chain Fatty Acids

WPMZ – Woody Plant Medium + Zeatin

Figures and Tables captions

1. Introduction

1.1 *Castanea genus and its importance*

Castanea genus belongs to the *Fagaceae* family and includes plants with a high variety of morphological and reproductive traits. *Castanea* trees have deciduous leaves and can reach 30 meters high.

This genus dates from the Tertiary period (between 65.5 My and 2.6 My ago) and suffered an East-Western dispersion from Japan to North America, forming the current distribution (Huntley & Birks, 1983). Genus fragmentation occurred by allopatric speciation, which resulted in the species that exist today (Lang *et al*., 2007). *Castanea sativa* Mill., the European chestnut native is widely distributed across the Mediterranean region, although in Portugal its presence is mainly in the North of the country (Casasoli *et al*., 2001; Fernández-López & Monteagudo, 2010). The genetic diversity of the European chestnut varies consistently in geographic distribution according to historical events, environmental variations and management practices. Although chestnut cultivation caused a consistent decrease in genetic variation, there are still regions acting as important natural sources of genetic diversity, that can be of great relevance for the study and the conservation of chestnut biodiversity and adaptive potential (Casasoli *et al*., 2001; Pereira-Lorenzo *et al*., 2016).

Chestnut is a multipurpose species, with economic importance both for nut and wood production. These features explain the need of further knowledge about this important genus for ecology and economy (Lang *et al*., 2007; Ji *et al*., 2018). The chestnut fruit is used in the food industry and in fresh market, while wood is used in furniture industry due to its good density. The tree itself protects the landscape for fixing the soil in slope zones and having an important ecological role in forestation and ecosystem services (Martin *et al*., 2012; Santos *et al*., 2015; Serrazina *et al*., 2015).

The nut of European chestnut shows the highest quality; in Portugal there are four denominations of protected origin – “Castanha da Terra Fria”, “Castanha da Padrela” “Castanha dos Soutos de Lapa” and “Castanha de Marvão-Portalegre”. The nut production is the major economic income for Trás-os-Montes region (Santos *et al*., 2017) and is one of the most exported dry fruits in Portugal. All these features explain the importance and need for in-depth knowledge of *Castanea sativa* Mill. and constitute a great motivation for genetic improvement of chestnuts.

1.2 *Castanea-Phytophthora cinnamomi host-pathogen interaction*

European forest species have been affected by Asian pathogens mostly due to the increase of world trading of plant material (Santini *et al*., 2013; Muller *et al*., 2016).

Phytophthora cinnamomi is a pathogenic oomycete that causes root rot (ink disease) and affects several tree species, like chestnuts, pines, oaks, and eucalyptus (Robin *et al*., 2012). The wide range of species affected by *P. cinnamomi* makes this pathogen an important threat for forestry and agriculture (Hardham, 2005; Robin *et al*., 2012). This pathogen infects the roots and cause serious injuries, extending the necrosis from the root to the collar (Santos *et al*., 2015). Besides the necrosis, *P. cinnamomi* causes also drying, yellowing and wilting leaves with smaller size and dieback (Frisullo *et al*., 2018).

Concerning infection, *P. cinnamomi* is a soil pathogen that can infect the host through zoospores, asexual structures, which are mobile in water and easily reach the plant root tissue (Hardham & Blackman, 2017). The zoospores penetrate non-lignified root tissue by releasing elicitins and disseminates through the stem. When susceptible host is infected, its defense genes are downregulated, allowing the easy growth of the pathogen (Oβwald *et al*., 2014). Root damage prevents water movement through the vascular bundle, reducing plant growth (Miranda-Fontaiña *et al*., 2007) by water and nutrient absorption deficit. Under infection, abscisic acid increases in roots, leaf water potential decreases and stomata closes, decreasing photosynthesis (Oβwald *et al*., 2014). This lack of water and nutrients caused by necrosis of the roots originates the death of the infected chestnut trees (Hardham & Blackman, 2017; Santos *et al*., 2015).

Mostly due to pests and diseases, the nut production decreased considerably during the XX century in the Southwest of Europe. In Portugal, the decrease in cultivation area was mainly due to the pathogen *Phytophthora cinnamomi* (Santos *et al*., 2015).

The coexistence of chestnut Asian species from Japan and China (*Castanea crenata* Sieb. & Zucc. and *Castanea mollissima* Blume) with *P. cinnamomi* (original from the Asian tropics) lead to the development of resistance against the pathogen during the evolution process, while North America and European species did not (Hardham, 2005; Lang *et al*., 2007; Oliva *et al*., 2013; Santos *et al*., 2017). Although the Asian species have proved resistance to ink disease, these species are not adapted to the Atlantic environmental conditions (Pereira-Lorenzo *et al*., 2010). Therefore, the resistance of Japanese and Chinese species to this pathogen led to their introduction in breeding programs over the last years as donors of resistance in controlled crosses (Costa *et al*., 2011; Santos *et al*., 2017).

The breeding programs using European and Asian *Castanea* species generate hybrids that potentially introduce the resistance to biotic stresses and also promote adaption to European environmental conditions and high quality of fruits (Serrazina *et al*., 2015).

The exotic pathogen introduction and climate change have increased substantially the need for the development of genetic tools to ensure conservation and improvement of tree genetic resources. In addition to obtention of new chestnut genotypes with improved resistance to *Phytophthora cinnamomi*, the understanding of the resistance increases and will allow, in the a near future, the identification of genes and regulators that will enable an expedite selection of improved genotypes for *P. cinnamomi* from the breeding program, assisted by molecular markers (MAS – Molecular Assisted Selection) (Santos *et al*., 2017).

1.3 *The genetics of resistance*

Resistance and tolerance are the two major mechanisms of plant defense. Resistance is the ability of the plant to limit pathogen multiplication and tolerance is the ability of the plant to reduce the effect of infection (Pagán & García-Arenal, 2018).
According to Santos *et al*. (2017), *Castanea* resistance is associated with diverse cellular processes. Putative genes that provide some insights about *Castanea* candidate resistance genes involved in *P*. *cinnamomi* response were identified. The presence of putative resistance proteins, cellulose synthase, regulation of gene expression and hormone signaling may be involved in the resistance to *P*. *cinnamomi*, as it was suggested by Serrazina *et al*. (2015) and Santos et al. (2017). Regulation of salicylic acid signaling and lignin synthesis by *Myb4* gene may also be involved in the chestnut resistance mechanism, in *C*. *crenata* and in hybrid resistant genotypes, avoiding pathogen progression (Santos *et al*., 2017). This way, the pathogen is limited in its growth by the host.

The secretion of antifungal proteins and the reinforcement of the cell wall are a part of the constitutive defense barriers to pathogen growth may explain the difference between *C. crenata* and *C. sativa* about *P. cinnamomi*'s resistance (Hardham & Blackman, 2010; Santos *et al*., 2017).
Also the secretion of toxic compounds is an effective chemical defense mechanism. According to Santos et al. (2017), Ginkbilobin-2 (Gnk2) is the most expressed protein, secreted by Ginkgo biloba seeds. It exhibits an antifungal activity (Wang & Ng, 2000; Sawano *et al*., 2007). *Cast_Gnk2-like* is the gene that better discriminates between susceptible and resistant genotypes. Cast_Gnk2-like may prevent pathogen growth

either by its chemical properties or by inducing Hypersensitive Response-related cell death (Santos et al., 2017) and has its highest expression in non-inoculation conditions, suggesting that the roots of *C. crenata* may be a hostile environment for *P. cinnamomi.* HR-related cell death is probably activated and cell walls may be reinforced in non-infected tissues, preventing further colonization (Santos *et al*., 2017).

Salicylic Acid (SA) is a compound that also induces defense responses against pathogens (Loake & Grant, 2007; Vlot *et al*., 2009). High concentrations of endogenous SA may induce Hypersensitive Response (Mur *et al*., 2008) and induce expression of *Cast_Gnk2-like* (Santos *et al*., 2017). According to Santos *et al.* (2017), if *P. cinnamomi* overcomes those chemical and physical barriers, the expression of specific pathogen recognition proteins is increased in the resistant genotypes.

Resistance is a quantitative trait (Irwin *et al*., 1995), which means that several genes are involved in the process, each one with a small contribution to the phenotypical variation and showing a Normal variation (continuous distribution). Each Quantitative Trait Loci (QTL) is a genomic region, constituted by one or more genes, involved in a quantitative trait. Most quantitative traits can evolve in response to selection; representing the additive variance a significant part of the phenotypic variance, which makes mutational selection important in the maintenance of quantitative trait variation (Barton & Keightley, 2002; Casasoli *et al*., 2005). Considering that quantitative traits are subjected to a strong environmental influence, it is difficult to select these characteristics from the indirect phenotype-genotype relation. The number of loci involved, the magnitude of their effects, the type of gene action (additivity, dominance, epistasis, and pleiotropy), and the existence of a genotype-environment interaction effect are the key factors of the evolution of adaptive traits (Casasoli *et al*., 2005).

Identification of QTLs associated with resistance in genetic linkage maps allows the use of molecular markers associated with genes of interest, making possible the identification and early selection of the most resistant genotypes, speeding up the selection process in breeding programs (Santos *et al*., 2017; Nishio *et al*., 2018).

1.4 *Molecular markers – Microsatellites*

Mendelian inheritance of alleles is a requirement for almost all population genetic analyses (Selkoe & Toonen, 2006). Molecular markers are useful tools for detecting genetic variation and provide a phenotype-genotype variation linkage (Varshney *et al*., 2005).

Molecular markers may be classified as hybridization-based markers (RFLPs), PCR-based markers (RAPDs, AFLPs, ISSRs and SSRs) and sequence-based markers (SNPs) (Varshney *et al*., 2007; Sehgal & Raina, 2008).

Microsatellites – Single Sequence Repeats (SSRs) are interesting molecular markers for use in eukaryotic organisms, with several applications in plant genetic research (Powell *et al*., 1996; Bandelj *et al*., 2004). SSRs are often used to classify and to identify genetic resources and varieties (Licea-Moreno *et al*., 2019), becoming a powerful technique for genetic studies (Morgante & Olivieri, 1993; Glenn & Schable, 2005; Selkoe & Toonen, 2006) and very important for breeding (Kalia *et al*., 2011; Parida *et al*. 2009).

The size of microsatellites is between 1 and 6 nucleotides and correspond to tandem repeats. They are found in the nuclear and organellar genomes of most taxa at high frequency (Pérez-Jiménez *et al*., 2013; Selkoe & Toonen, 2006). SSRs constitute a large fraction of non-coding DNA but many recent reports have identified a large number of SSRs located in transcribed regions of the genomes (Kalia *et al*., 2011; Santos *et al*., 2015). SSR have the potential to affect genetic function, depending on their location. A microsatellite located in a coding region can affect the activation of a gene and therefore, the expression of a protein (Kalia *et al*., 2011). If located in a noncoding or genic region, the microsatellite may impact gene regulation or gene transcription respectively (Lawson & Zhang, 2006) and if located in promoter regions, microsatellites may affect gene activity (Li *et al*., 2002). The variation in the microsatellite size within a gene can ultimately lead to phenotypic changes (Li *et al*., 2004) and may be a source of variation fitness-related traits (Kashi *et al*. 1997; King & Soller 1999; Trifonov, 2002). This size variation may be important for population adaptation and survival (Li *et al*. 2000, 2002).

Genotyping of plants of commercial value becoming frequent and important. As SSR markers are very polymorphic due to its high mutation rates (Guichoux *et al*., 2011), abundance and variability in most of locus (Li *et al*., 2002; Bandelj *et al*., 2004), made it a powerful tool for genotyping (Weising et al*.,* 1995; Staub & Serquen, 1996; Ruane & Sonnino, 2007; Kalia et al*.,* 2011). Several techniques have been developed in order to take advantage of the hypervariable nature of microsatellites for applications in plant genomics. This nature might be explained by single-stranded DNA slippage, double-stranded DNA recombination, mismatch/double strand break repair and retrotransposition (Kalia et al, 2011).

SSRs have become of very common use due to their repeatability, transferability, relative low costs, abundance in eukaryotic genomes, variability, high information content, Mendelian co-dominant inheritance and mutational behavior characteristic (Kelkar *et al*., 2010; Morgante & Olivieri, 1993; Powell *et al*., 1996; Weising *et al*., 1997). When species

are genetically close, SSR markers can be transferred across the taxa, which is commonly known as ‘‘transferability’’ (Ellis & Burke, 2007; Varshney *et al*., 2007).

SSR are being used for diversity studies: genotyping, genome mapping, breeding programs, linkage analyses, gender identification, parentage analyses, fingerprinting, marker-assisted selection, population structure assessment, evaluation of taxonomic and phylogenetic relationships and pedigree analysis (Ellegren, 2004; Jones *et al*. 2010; Kalia *et al*., 2011; Mittal & Dubey, 2009; Parida *et al*. 2009; Powell *et al*. 1996). These markers can also be used for the construction of genetic linkage maps and QTL analysis, allowing the identification of loci and candidate genes linked to the traits of interest (Neeraja *et al*., 2007; Romero *et al*., 2009). Their use increased since the late eighties for applications such as genomic distribution, evolutionary dynamics, biological function (Guichoux *et al*., 2011; Varshney *et al*., 2007).

Chloroplast Simple Sequence Repeats (cpSSRs) have been extensively used to study genomic variations in plants and gene flow in natural populations (Provan *et al*., 2001) due to their uniparental inheritance (Kalia *et al*., 2011).

In the Japanese chestnut, more than 300 simple sequence repeat markers (SSRs) have been developed, showing high transferability to other *Castanea* species (Nishio *et al*., 2011; Kubisiak *et al*., 2013), allowing the alignment of the genetic maps of Japanese, Chinese and American chestnuts. European chestnut maps were constructed using an interspecific crosses (Barreneche *et al*., 2004; Casasoli *et al*., 2001, 2005; Santos *et al*., 2017) but have not been sufficiently connected to these other maps (Nishio *et al*., 2018).

Although microsatellites may be transferable across taxa, they are in general species-specific, therefore cross-contamination by non-target organisms is a much less problem compared with techniques that employ universal primers that can amplify DNA from any species (Selkoe & Toonen, 2006).

1.5 *Micropropagation*

Micropropagation provides an adequate method for rapid mass propagation of selected genotypes, especially in recalcitrant species as it is the case of chestnut. *In vitro* recalcitrance is the inability of plant cells/tissues to respond in culture, being a limiting factor for the manipulation and multiplication of the species (Benson, 2000).

Clonal propagation is genotype dependent, for *in vitro* establishment and multiplication rates (Miranda-Fontaíña & Fernández-López, 2001).

Micropropagation is important because even if a hybrid is resistant, if it is not capable of multiplication, it is useless.

Micropropagation technique is a quick way to multiply an individual, producing thousands of clones from plant cells or tissues in sterile conditions with controlled temperature and photoperiod.

To micropropagate selected genotypes, is required the optimization of culture media for *in vitro* establishment and multiplication is required (Selkoe & Toonen, 2006). Rooting stage can also be a limiting part of the process, for some species. By the technique used in this thesis, rooting is done already *ex vitro*.

During the acclimation process, the humidity decreases progressively, allowing the plant to adapt to the new environment and the stomata starts functioning and controlling the water loss by evapotranspiration (micropropagated plantlets do not have control over their stomata because the container's environment is always saturated with humidity). The acclimation phase that occurs in a greenhouse, is the most challenging step for micropropagation of woody species. It occurs before transfer to the field, where the irradiance is much higher and the humidity much lower, comparing to the pots (Pospóšilová *et al*., 1999).

In Portugal, several studies were made in chestnut, from *in vitro* propagation by Gonçalves *et al*. (1994, 1998), to genetic transformation by Seabra & Pais (1998) and phenotyping and genotyping of chestnut hybrids by Santos *et al*. (2015) and Santos *et al*. (2017), respectively.

The chestnut breeding program for resistance to biotic stresses on course is based on controlled-crosses made between the European chestnut and the Asian species resulting in two full-sib pedigrees, SC (*C. sativa* x *C. crenata*) and SM (*C. sativa* x *C. mollissima*) (Costa *et al*., 2011). The most resistant genotypes to *Phytophthora cinnamomi*, the causal agent of root rot, were further micropropagated.

1.6 *Objectives*

In this work, a hybrid progeny obtained from crosses between *C. crenata* and *C. sativa* was phenotyped and genotyped in respect to *P. cinnamomi* susceptibility and to determine an association phenotype-genotype.

The specific objectives of this work were:

i) micropropagation of the most resistant genotypes, selected from the Chestnut breeding program INIAV; the data of multiplication and rooting is important for the project on course, where this thesis fits,

ii) phenotyping and genotyping the progeny of a controlled cross between *C. sativa* x *C. crenata* and

iii) try to correlate the phenotype and the genotype, by associating hybrids DNA polymorphisms to its level of susceptibility/resistance to *P. cinnamomi*.

In a more general objective, this work will contribute to the research program on course at INIAV, by selecting new genotypes with improved resistance to *Phytophthora cinnamomi*, to be used as rootstocks, for grafting of national varieties for nut production.

2. Materials and Methods

2.1 *Plant Material*

Different plant material was used depending on the task. Micropropagation and rooting was performed for the four main genotypes, previously selected: SM904, SC55, SC1202 and SC914. SC55 was selected from the controlled cross of 2006, SM904 and SC914 were selected from the controlled cross of 2009 and SC1202 was selected from a controlled cross from 2012.

For phenotyping, a hybrid progeny of controlled crosses performed in 2016 were used, with *C. sativa* as the female parent and *C. crenata* as male parent: the families BAC, BCC and BDC wherein Portuguese variety Bária as female parent and the family CFC wherein Portuguese variety Colarinha as the female parent. *C. crenata* was the pollen donor and the crosses were made with a mixture of pollen of two *C. crenata* trees, in order to increase the probability of fertilization.

The hybrids were obtained by germinating nuts result of controlled pollination.

All the plants phenotyped were four years old.

2.2 *Methods*

2.2.1 *Micropropagation*

Three stages of micropropagation were performed: multiplication – using the medium MSB (Murashige & Skoog + Benzilaminopurine (0,1 mg/L) medium (S1 Table of Supplementary Information)), elongation – using the medium WPMZ (Woody Plant Medium + Zeatin (0,1 mg/L) medium (S2 Table of Supplementary Information)) and pre-rooting – using AC (Activated Charcoal (0,3%) medium (S3 Table of Supplementary Information)).

The multiplication medium (Murashige & Skoog, 1962) was supplemented with benzilaminopurine (BAP), which is a cytokinin that promote the multiplication, while the elongation medium (Lloyd & McCown, 1980) has Zeatin, that is a natural cytokinin that induces more vigor to the shoot. Shoots were subcultured every 6/8 weeks and the used medium alternated between MSB and WPMZ.

Pre-rooting medium has no hormones, in fact, activated charcoal is added to prepare the shoots for the auxin hormone for rooting (Barve & Mehta, 1993). Shoots stayed 5 to 7 days in this media before being rooted.

The shoots were kept in a chamber with controlled temperature, humidity and photoperiod, at approximately 23°C and 62% humidity, with a photoperiod of 16 hours of light and 8 hours of darkness.

Rooting is done under *ex vitro* conditions, and therefore also corresponds to acclimatization, being an adaptation of humidity and temperature conditions. Shoots were subjected to hormonal shock for 1 minute with IBA (Indolbutiric Acid, 1g/L), an auxin that promotes the root development, in a process called dipping (Gonçalves *et al*., 1994, p.304) and were transferred to paperpots with substrate to produce roots.

For calculation of rooting percentages, plantlets that grew roots were counted and divided by the total number of plants, in each genotype. 405 plants from SM904, 89 plants from SC55, 87 plants from SC1202 and 227 plants from SC914 were used.

Both multiplication rates and rooting percentages were calculated, to evaluate the four selected genotypes, SM904, SC55, SC1202 and SC914. Multiplication rate was calculated for each genotype, 75 shoots (5 boxes with 15 shoots each, per genotype) were used in total, with 3 replicates. After 6 weeks the shoots were counted (if in the place of 1 shoot there are 4, the multiplication rate is 3); only MSB media was used for this assay.

Average and standard deviation of multiplication rate are presented in S5 Table. ANOVA and Tukey test were also performed, in S6 and S7 Tables of the Supplementary Information, respectively. Both multiplication rates and rooting percentages were

statistically analyzed by GraphPad Prism 6 software. Average and standard deviation of rooting percentage were performed in S8 Table in the Supplementary Information. ANOVA was also performed and is presented in S9 Table in the Supplementary Information.

2.2.2 *Inoculation*

Inoculation of roots with *P. cinnamomi*

To accomplish root inoculation, it is necessary to use a fresh culture of *P. cinnamomi*, which was prepared by transferring a small piece of mycelium from a previous culture to a new fresh PDA (Potato Dextrose Agar) nutritive selective medium with incubation at 24°C in the darkness for 4-5 days (Figure 1).

Figure 1 – Petri dish with five days PDA culture of *Phytophthora cinnamomi.*

Vermiculite moisturized with the V8 solution (S4 Table of the Supplementary Information) was prepared and autoclaved twice during 20 minutes at 121ºC/1 bar. Mycelium of *P. cinnamomi* was mixed into this nutritive medium and incubated at 24ºC in darkness for three weeks.

The *P. cinnamomi* inoculum added was 10% of the volume of each plant pot, approximately 20 g were mixed in 300 mL of each plant pot soil (Figure 2).

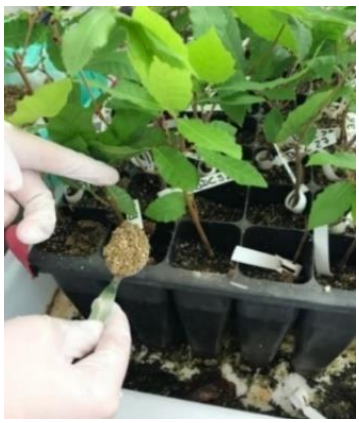


Figure 2 – Inoculation of plants with *P. cinnamomi.*

Confirmation of *P. cinnamomi* presence in the soil

The re-isolation of *P. cinnamomi* in the soil was performed using the Baiting Method, described in Erwin & Ribeiro (1996), proofing that the plants soil was successfully inoculated.

During the time of inoculation, pieces of leaves from susceptible species were placed in the water of the inoculated plants every two weeks. After 3-5 days, the leaves were observed under the microscope to find *P. cinnamomi* morphological structures. The aim of this procedure is checking if *P. cinnamomi* was active during the essay.

2.2.3 *Plant Phenotyping*

The plant phenotyping of the progenies under study evaluated its susceptibility to *P. cinnamomi* by observation of necrotic root lesions and progression of external stem lesions after root inoculation with *P. cinnamomi*. Plants of *C. sativa* and *C. crenata* were used as control.

One hundred days after inoculation the plants were removed from the soil and were checked for lesions according to Santos *et al*. (2015). The root lesion level was determined (Table 1) and the external lesion was measured according to the formula

$$\%\ \text{progression} = \frac{\text{Lesion lenght (cm)}}{\text{Plant lenght (cm)}}$$

. The survived hybrids were potted again and were kept in the greenhouse, in the same conditions.

For phenotyping, 13 plants from BAC, 20 from BCC, 60 from BDC and 20 from CFC were used, in a total of 113.

Table 1 – Scale to evaluate the root lesion level due to *P. cinnamomi* infection by Santos *et al*. (2015).

Root Lesion Level	Progression
1	No root lesion observed
2	0,1-9,9% root lesion
3	10-19,9% root lesion
4	20-29,9% root lesion
5	30-49,9% root lesion
6	>50% root lesion

2.2.4 *Plant Genotyping*

Although phenotyping was done for all four crosses, genotyping was made only in one, BDC progeny. For 47 BDC individuals and the parents, *C. sativa* (variety Bária) and both *C. crenata* trees (C1 and C2), DNA was extracted and plants were genotyped as described below.

DNA extraction

200 mg of leaf tissue were grinded with a mortar and pestle in liquid nitrogen and divided per 2 tubes for DNA extraction using cetyl Trimethylammonium bromide (CTAB) Protocol (Annex 1 in Supplementary Information). The agarose gel with the DNA samples of each genotype is present in Figure S6 of the Supplementary Information. The concentration of DNA used was 5 ng/µL.

DNA purification

A volume of 20 µL of DNA sample was further purified from DNA extraction soluction using MicroElute® DNA Clean Up Kit Centrifugation Protocol (Annex 2 in Supplementary Information).

Evaluation of DNA concentration and quality

After DNA extraction, Qubit was used for determination of dsDNA concentration (ng/µL) of each sample and the NanoDrop was used to assess DNA purity, A260/A280 nm and A260/A230 nm ratio determination (S11 Table of the Supplementary Information).

SSR PCR Amplification

A total of 16 microsatellite primer-pairs were used: CcPT0003 (Cc3), CcPT0011 (Cc11), CcPT0013 (Cc13), CcPT0014 (Cc14), CcPT0025 (Cc25), CcPT0030 (Cc30), CmSI0003 (Cm3), CmSI0361 (Cm361), CmSI0433 (Cm433), CmSI0510 (Cm510), CmSI0537 (Cm537), CmSI0550 (Cm550), CmSI0559 (Cm559), CmSI0593 (Cm593), CmSI0691 (Cm691) and CmSI0735 (Cm735), for amplification reactions. These molecular markers used for genotyping were Expressed Sequence Tag microsatellites (EST-SSRs) (Santos *et al*., 2015) and were used to amplify certain genes potentially associated with *P. cinnamomi*'s resistance. The CcPT primers were designed from transcriptomes of *C. crenata* by Santos *et al*. (2015) and the CmSI were designed in transcriptomes of *C. mollissima* by Kubisiak *et al*. (2013). All primers were selected from transcriptome of genotypes inoculated with *P. cinnamomi*. The primer sequences (5'-3') is in Table 2.

Table 2 - Sequences (5'-3') of the 16 primer-pairs used, CcPT (designed from transcriptomes of *C. crenata* by Santos *et al*., 2015) and CmSI (designed in transcriptomes of *C. mollissima* by Kubisiak *et al*., 2013).

Loci	Primer forward sequence (5' - 3')	Primer reverse sequence (5' - 3')
CcPT_000**3**	GGTGCCCAGATTTACGAGAA	GTTTCTTATCGCTTGGAGTCACAGCTT
CcPT_00**11**	TCATCCAAGAAGCCCTCAAC	GTTTCTTTTCTGCCTCTTTTGTTGCCT
CcPT_00**13**	AGTACGTAGTCGAAGAGAGAAGAG	GTTTCTTAGTGAAGTTTTGTCGGGGTG
CcPT_00**14**	AGGCGCATTCAAAGAAAGAA	GTTTCTTAGCTGATCAACTCTCGCCAT
CcPT_00**25**	GGGTCGGAATACATGTGACC	GTTTCTTGCTTTGATCCAACCAACGAT
CcPT_00**30**	TCGAGGCTTCTTGTTCCACT	GTTTCTTGAATTGGTGGAGGCAGAAAA
CmSI000**3**	CATCCCTGATCCGTTCTTC	GTTTCTTTCAAAGGTGGGTCTTTGAGG
CmSI0**361**	GCCAACAACTGTGGCTTCTT	GTTTCTTTGTCGAAGTAGAGGATGCTTCA
CmSI0**433**	CTCCTCCATTTCCCAATTCA	GTTTCTTCGCTTGTGGTACATCTTGGA
CmSI0**510**	ACGGCTTACAAAGAAACACA	GTTTCTTAAGAAGGTCGTCGATCTGGA
CmSI0**537**	GAGATGGGTTGGGAAGGTT	GTTTCTTGGCCTCTCTGGTTTGTGTGT
CmSI0**550**	TACGAAGATGGAGAGAAGGCA	GTTTCTTTTTGGTTGACAACTGGGTCA
CmSI0**559**	TAGGTGGGAGTGAAGGTGTTG	GTTTCTTTATCTCGCTCGCTCCATCTT
CmSI0**593**	TAAAGGAGCTCCACGGAAGA	GTTTCTTAAGGATGCTCCTAGAGCTGAGA
CmSI0**691**	GTTTCTTGGCTGCTGTTGCTGTACTTG	GTTTCTTGGCTGCTGTTGCTGTACTTG
CmSI0**735**	ACGCCTTCAGTTGCTGTTTC	GTTTCTTCAACGGTCTTACCCTTTGGA

Amplification Protocol

The PCR mixtures were prepared for each primer, using the protocol described in Annex 3 of Supplementary Information. The PCRs were carried out using a thermocycler (Biometra). Amplified fragments were separated in an agarose gel (Figure S7 of the Supplementary Information).

Agarose Gel Electrophoresis

The agarose gel was prepared and visualized under UV light and the image acquisition was made with a BIO-RAD Gel-Doc equipment (Figures S6 and S7 of the Supplementary Information).

Capillary Electrophoresis

The fragment analysis of SSR amplified fragments was done for 47 genotypes with 16 primers, labeled with 2 dyes (NED-yellow and VIC-green) and 500 ROX™ as Size Standard (red) by capillary electrophoresis, in 3500 Genetic Analyzer (Applied Biosystems). The protocol is described in Annex 5 of the Supplementary Information.

Genotype calling

After capillary electrophoresis, the peak values of each primer pair were scored reading the molecular weight (3500 Genetic Analyzer, Applied Biosystems) and allelic frequencies determined (Figure S8 in the Supplementary Information).

Data analysis

Allelic frequencies were obtained by creating five groups, joining the hybrid genotypes according to the phenotype of each one. The groups were: hybrids with no symptoms ("nosympt"), hybrids with level 2 and 3 of root rot ("rootrot23"), hybrids with level 4 and 6 of root rot ("rootrot46"), hybrids with lesion ("lesion") and the hybrids that died ("dead"). The allelic frequency of each group was calculated by dividing the number of individuals with a given allele by the total number of individuals. Heterozygosity observed (*Ho*) and expected (*He*) were also calculated per locus. *Ho* was calculated with the number of

observed heterozygous individuals divided by the total number of individuals. *He* was calculated using the formula $1 - \sum(allelic\ frequencies)^2$.

The allelic frequencies of each group were used in PopTree software to generate a Neighbor-Joining tree, that joins the groups with more alleles in common. Neighbor-Joining tree is an unrooted tree, with no phylogenetic basis, just used to group according to similarity.

GraphPad Prism 6 software was used to show the results in graphics.

The primers used amplified SSR present in candidate genes for resistance. The prediction of gene, gene function, gene regulation and identity (%) was obtained by Primer-BLAST and UniProt through each primer-pair sequences.

A selection of the alleles present only in the groups with no symptoms and with root rot was made. The sum of the frequencies obtained for group without symptoms together with group with levels 2 and 3 of root rot were considered as putative alleles involved in resistance.

3. Results

3.1 *Micropropagation*

The four genotypes, selected from the breeding program for resistance to *P. cinnamomi* were micropropagated and rooted. Figure 3 shows the different stages from multiplication/elongation and pre-rooting of micropropagation (Figure 3 a and b) to *ex vitro* rooting (Figure 3 c).

a) **b)** **c)**

Figure 3 – Different steps of micropropagation process of selected chestnut genotypes - a) multiplication/elongation in medium with cytokinin; b) pre-rooting in medium with

activated charcoal; c) rooting in paperpots with substrate, immediately after the dipping with IBA (auxin).

The multiplication rate average obtained is genotype dependent (Figure 4). The genotype SM904 presented the highest multiplication rate with a mean value and standard deviation of 2.543 ± 0.617, while the genotype SC914 showed the lowest value, with a multiplication rate and standard deviation of 1,908 ± 0,328. The calculations for multiplication rate are presented in detail in the Supplementary Information, with the average and the standard deviation obtained per genotype in S5 Table and ANOVA and Tukey test in S6 and S7 Tables, respectively. These statistical analyses showed significant differences between the genotypes (ANOVA) ($P < 0.0001$). The highest difference was observed between SM904 and SC914 genotypes (Tukey test) ($\alpha=0,05$).

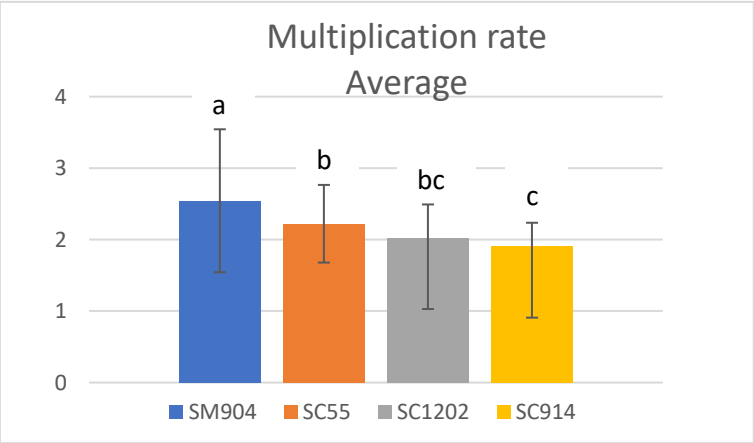


Figure 4 – Average multiplication rate and standard deviation, obtained per genotype (n=75) after 6 weeks (letters a, b and c shows the differences between the genotypes, according to the Tukey test on S7 Table in the Supplementary Information).

Rooting was also genotype dependent (Figure 5) with SM904 presenting the highest rooting percentage (79.6% ± 4.28), while SC914 presented the lowest value (61.2% ± 9.37). ANOVA revealed that differences were not statistically significant ($p = 0.2263$). The rooting percentage is presented in detail in Supplementary Information, with the average and the standard deviation obtained per genotype in S8 Table and ANOVA

presented in S9 Table. ANOVA showed no significant differences between the genotypes, regarding rooting percentage.

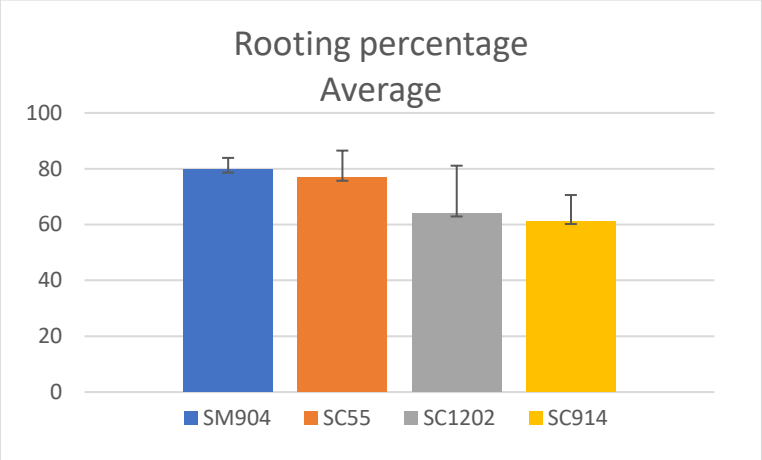


Figure 5 – Average rooting percentage observed per genotype and standard deviation.

Multiplication rate and rooting percentage were highly correlated, with 93,19 % (Figure 6), showing that the genotypes that presented higher multiplication were also those most prone to rooting.

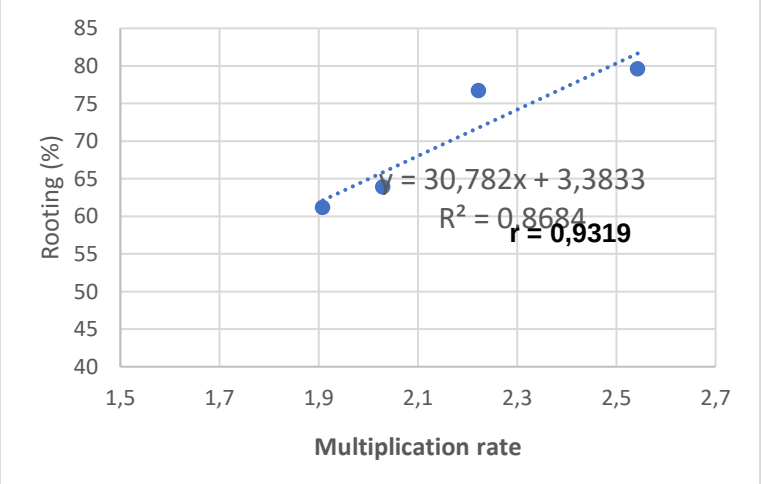


Figure 6 – Correlation between multiplication rate and rooting percentage.

3.2 *Phenotyping*

The symptoms of *Phytophthora cinnamomi* infection were recorded as root rot and external lesion 100 days after inoculation (S10 Table of the Supplementary Information). Regarding the symptoms observed, it is possible to see the difference between the roots of healthy plants, that were white (Figure 7a) while the roots of infected plants were dark and presented signs of root rot (Figure 7b).

Figure 7 – Phenotyping evaluation in four-year old plants, 100 days after inoculation with *P. cinnamomi*. Healthy plant, with no signs of infection/root rot (a). Root rot (b).

Stem external lesion could also be observed (Figure 8), as well as external and internal lesions of both stems and roots, highlighted with a red arrow in Figure 9. The measures of both stem and root lesions are presented in the S10 Table of the Supplementary Information.

Figure 8 – External lesion in a four-year old chestnut plant, a symptom caused by *P. cinnamomi* infection; the lesion is measured as described in *Methods*.

Figure 9 – Four-year old plants with external and internal lesions in stems and roots, a result of *P. cinnamomi* inoculation.

After 100 days of inoculation only 6% of the inoculated plants of progeny BDC died (Figure 10). During the phenotyping assay, the plants were daily monitored for evaluating the progression of the disease, as shown in the Figure 11. In this figure it is possible to observe that the symptoms of infection became visible approximately after 27 days and they presented the highest level between day 33 and day 48, rising 31,3%.

Controls of *C. sativa* and *C. crenata* plants were used. While *C. sativa* showed lesion of the collar and died, *C. crenata* showed just intermediate root rot (S10 Table of the Supplementary Information).

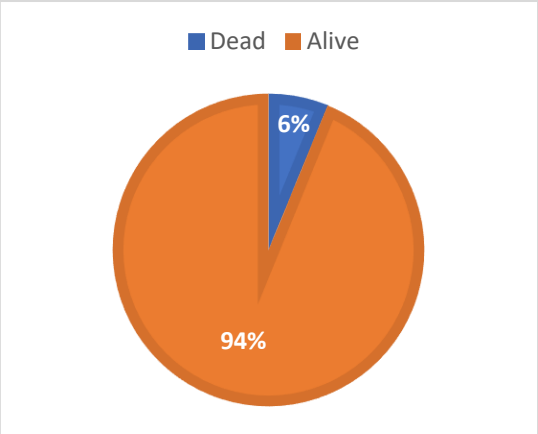


Figure 10 – Percentage of the total chestnut survival plants (orange) after the 100th day of inoculation with *P. cinnamomi*.

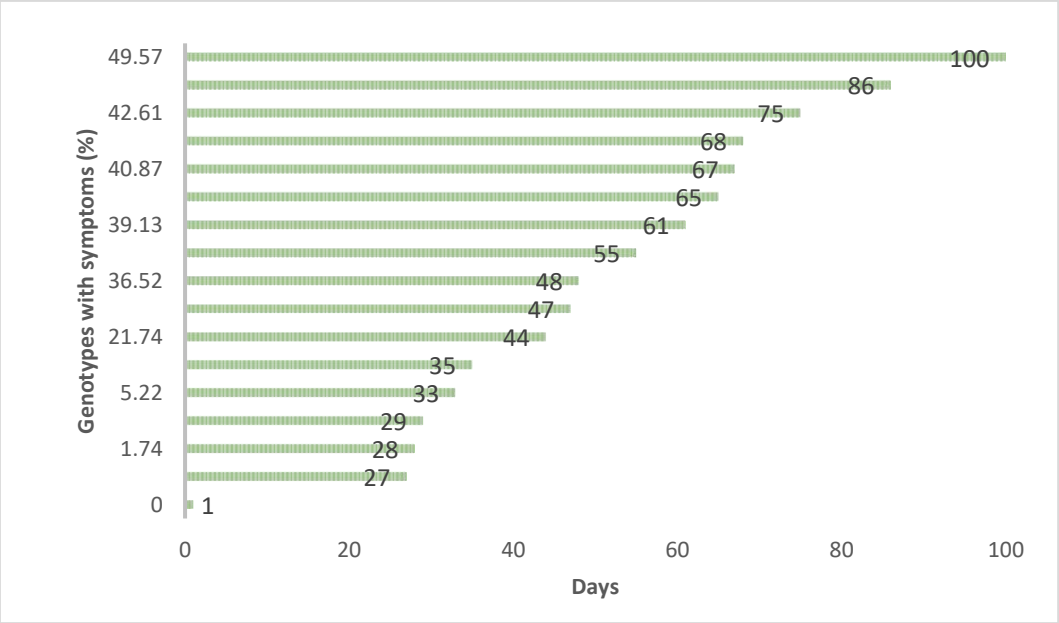


Figure 11 – Percentage of chestnut plants subjected to phenotyping, with symptoms of root rot observed during the 100 days period after inoculation with *P. cinnamomi*. Progenies BAC, BCC, BDC and CFC were subjected to the phenotyping evaluation.

Several plants were asymptomatic and between 60% and 70% of the plants of each genotype showed no symptoms after the 100 days essay (Figure 12).

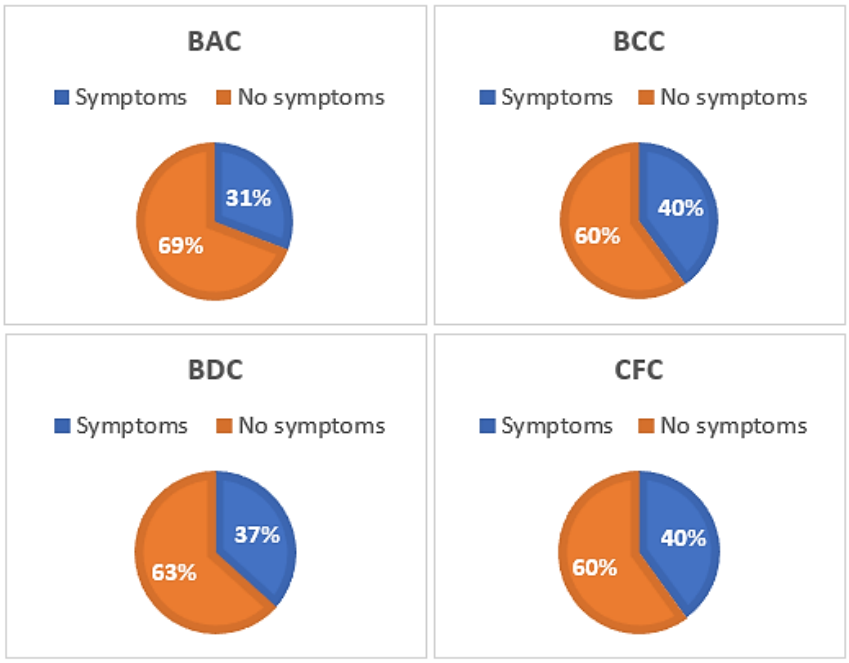


Figure 12 – Percentage of inoculated plants with and without symptoms caused by *P. cinnamomi* infection, observed in each cross.

Furthermore, a high percentage of plants, 66%, showed less than 20% of root rot (Figure 13).

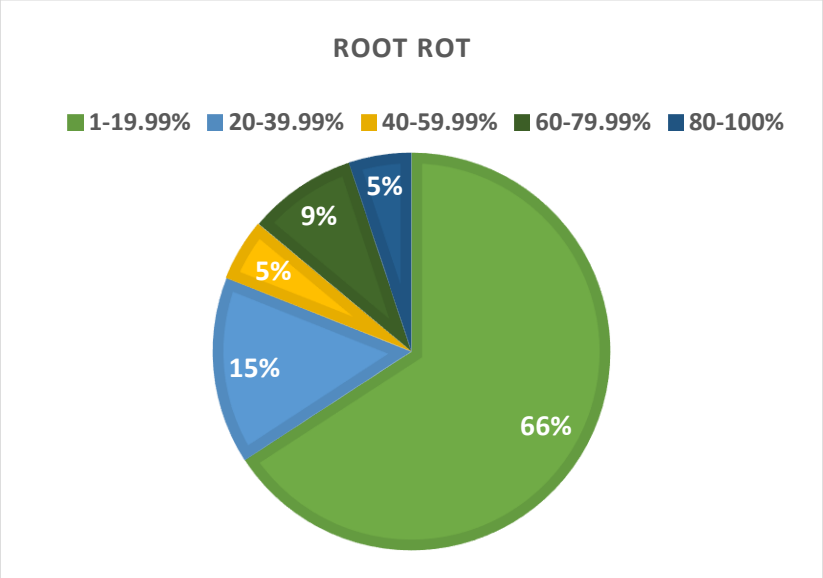
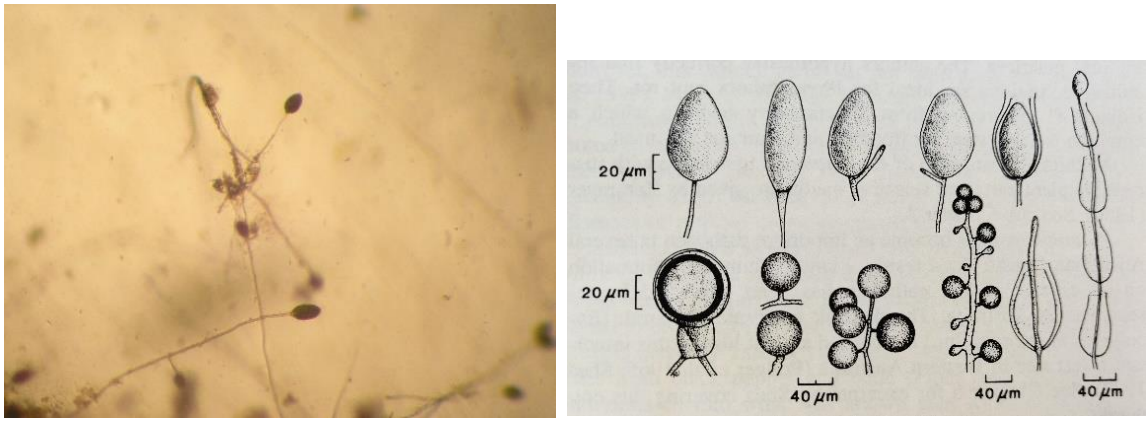


Figure 13 – Percentage of chestnut plants with root rot in each level: 1-19,99%, 20-39,99%, 40-59,99%, 60-79,99% and 80-100%.

After the inoculation process, it was possible to re-isolate and confirm that the strain used was *P. cinnamomi*. Through the observation of morphological characteristics of the sporangia and the formed zoospores, we could determinate the presence of the pathogen in leaves left in the irrigation water, several times during the assay, a test used for control of presence of the pathogen. Figure 14 shows the *P. cinnamomi* morphological structures found in the assay (a) and the typical structures observed in the literature (b).



a) b)

Figure 14 – *P. cinnamomi* sporangia as a confirmation test of the pathogen presence in the phenotyping assay (a); morphological characteristics of *P. cinnamomi*, adapted from Erwin & Ribeiro (1996) (b).

3.3 *Genotyping*

DNA fragment analysis was performed for genotyping the progeny. S12 Tables of the Supplementary Information shows the values of the allele peaks observed for each locus/genotype and Figure S7 shows the alleles of two heterozygous individuals, as an example of how the allele sizes were scored.

Observed heterozygosity (*Ho*) and expected heterozygosity (*He*) were calculated per locus (Table 3). Primer Cm3 presented the lowest observed and expected heterozygosity (*Ho*=0.106 and *He*=0.101) and the primer Cm559 the highest observed and expected heterozygosity (*Ho*=0.936 and *He*=0.774) (Table 3 and Figure 15).

Table 3 – Characterization of 16 EST-SSR markers: number of alleles (NA), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and allele size average (Al. size av., in bp). Number of alleles and heterozygosity were estimated for chestnut samples.

Loci	Alleles	*Ho*	*He*	Allele size average (bp)
CcPT_000**3**	4	0,854	0,533	426
CcPT_00**11**	4	0,362	0,247	431
CcPT_00**13**	7	0,234	0,671	197
CcPT_00**14**	3	0,851	0,5811	172
CcPT_00**25**	4	0,66	0,53	352
CcPT_00**30**	7	0,894	0,722	488
CmSI000**3**	2	0,106	0,101	236
CmSI0**361**	4	0,787	0,232	283
CmSI0**433**	3	0,574	0,439	181
CmSI0**510**	5	0,766	0,55	281
CmSI0**537**	4	0,766	0,533	163
CmSI0**550**	5	0,553	0,7254	305
CmSI0**559**	6	0,936	0,774	143
CmSI0**593**	4	0,745	0,545	315
CmSI0**691**	3	0,894	0,502	183
CmSI0**735**	5	0,468	0,659	140

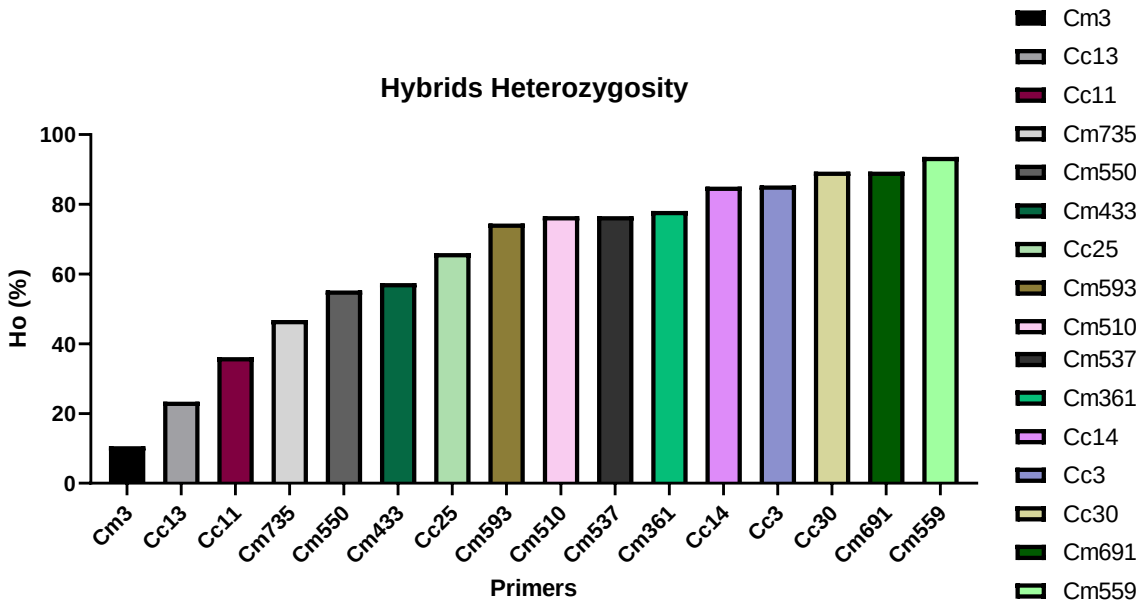


Figure 15 – Percentage of observed heterozygosity per primer, for BDC progeny.

The primers were also used to study the paternity, as a mixture of pollen of 2 male parents was used in the controlled pollinations. Alleles obtained for each hybrid were compared with the alleles of the male parents for paternity test (S12 Tables). For 68% of the genotypes, it was possible to determinate the male, as 57% of the genotypes have C1 male parent and only 11% of the genotypes have C2 as the male parent. The other 32% that had no correspondence, primers did not distinguish between the two male, the result was inconclusive (Figure 16).

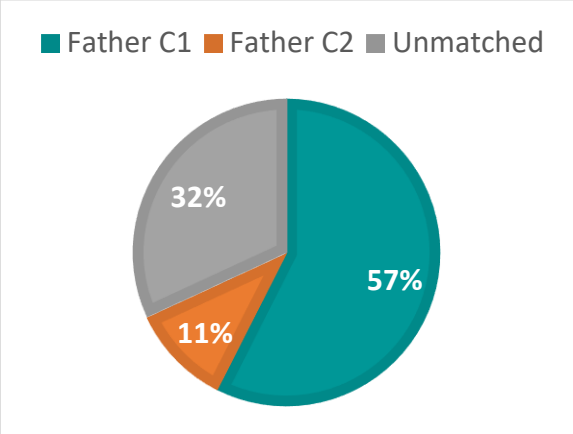


Figure 16 – Percentage of genotypes of the chestnut progeny with *C. crenata* 1 (C1) as the male parent, *C. crenata* 2 (C2) as the other male parent and those whose male parent could not be determined.

By analyzing the alleles of the two possible male parents (C1 and C2), some conclusions could be made about the paternity. The primers that could distinguish between the two male parents were the following: Cc25, Cc30, Cm3, Cm433, Cm550, Cm559, Cm593 and Cm735, as shown in the Figure 17. The primer that revealed higher HPDP percentage (Hybrids whose Paternity is Distinguished by a given Primer) was Cc30, with 57,4% of the hybrid's paternity distinguished.

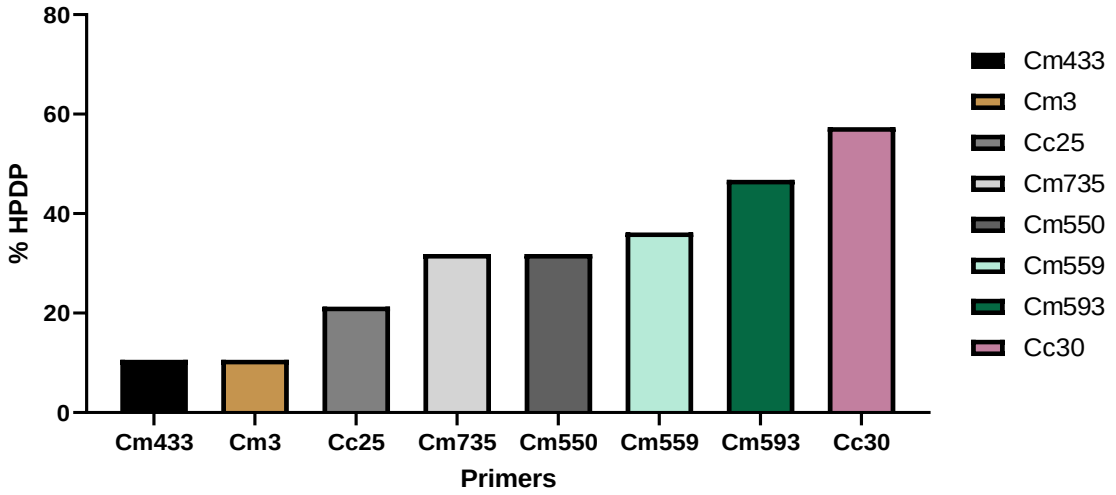


Figure 17 – Percentage of hybrids whose paternity was possible to determine by each primer.

The information obtained for predicted amplified gene, predicted gene function, gene regulation and identity (%) for each locus is presented in Table 4. Some primers amplified regions that were considered more important for their candidate relation with the processes of pathogen's defense. Those primers are Cc3, Cc11, Cc14, Cc30, Cm3, Cm537, Cm691 and Cm735, highlighted in blue.

Locus Cc3 is related to lateral roots and leaves development and the gene is upregulated in the presence of a wound. The predicted gene amplified by primer Cc11 is upregulated under stress in response to hypoxia. Cc14 is linked to inflammatory response and fatty-acid accumulation. The predicted gene amplified by the primer Cc30 is related to plant development and stress response, increasing auxin production. Cm3 is related to defense response against biotic stress. Primer Cm537 has the predicted amplification of the gene corresponding to protein PAT-1 homolog 2-like, related to increasing immune response. Cm691 and Cm735 are loci related auxin, hormone that promotes roots development. Both primers Cc11 and Cc30 have a predicted amplification of *Patellin-3-like*, that is involved in the regulation of auxin, just like Cm691 and Cm735.

Table 4 – Predicted amplified gene, predicted gene function, gene regulation and identity (%) of each used locus (the loci marked in blue have candidate relation to the processes of pathogen's defense).

Loci	Predicted Gene	Predicted Gene function	Gene regulation	Identity (%)
CcPT_000**3**	AP2/ERF and *B3 domain-containing transcription factor RAV1-like*	Ethylene-activated signaling pathway, lateral root development, leaf development	Upregulated in the presence of a wound	100
CcPT_00**11**	*Patellin-3-like* and protein EXORDIUM-like (*Quercus suber*)	Response to hypoxia (EXORDIUM-like) Regulators of auxin (Patellin-3-like)	Upregulated in stress (tolerance)	100
CcPT_00**13**	NONE			
CcPT_00**14**	*Peroxisomal acyl-coenzyme A oxidase 1-like* (*Quercus suber*)	Lipid/fatt acid metabolic process and oxidation, oxidorreductase, Jasmonic acid biosynthesis, peroxisome	Upregulated in the presence of fatty-acid accumulation	100
CcPT_00**25**	NONE			
CcPT_00**30**	*Patellin-3-like* (*Quercus suber*)	Plant development and stress response	Its upregulation upregulates the auxin-mediated PIN1	100
CmSI000**3**	CCG-binding protein 1 (*Quercus suber*)	Mediator complex binding, pollen tube guidance, defense response to fungus, embryo development ending in seed dormancy	Upregulated in the presence of biotic stress	100
CmSI0**361**	Zinc finger A20 and AN1 domain-containing stress-associated protein 8-like and Naringenin,2-oxoglutarate 3-dioxygenase (F3H) (*Quercus suber*)	Involved in environmental stress response Secondary compounds biosynthesis	Upregulated in roots and fruits during winter dormancy Upregulated under abiotic stresses	100
CmSI0**433**	60S ribosomal protein L6-1-like (*Quercus suber*)	Cytoplasmic translation, ribosomal large subunit assembly, RNA binding, structural constituent of ribosome		100
CmSI0**510**	*Quercus suber* uncharacterized LOC111997446			100
CmSI0**537**	Protein PAT1 homolog 2-like (*Quercus suber*)	RNA binding, negative regulation of translation	mRNA decay via PAT1 may rapidly instigate immune responses	99
CmSI0**550**	SPX domain-containing membrane protein At4g22990-like (*Quercus suber*)	Transmembrane transport - mediates Pi (phosphate - macronutrient) influx to the vacuoles		92
CmSI0**559**	*4-hydroxybenzoate polyprenyltransferase, mitochondrial-like* (*Quercus suber*)	Isoprenoid biosynthetic process, ubiquinone (CoQ) biosynthetic process - involved in mitochondrial respiratory chain		100
CmSI0**593**	Uncharacterized LOC111988154 (*Quercus suber*)			100
CmSI0**691**	*Auxin response factor 19-like* (ARF19) (transcription factor)(*Quercus suber*)	Auxin-activated signaling pathway, lateral root development	Auxin increase, upregulates the ARF	98
CmSI0**735**	Protein scarecrow-like (SCR) (*Quercus suber*)	Regulation of transcription, DNA-templated, root development	Expressed predominantly in the roots, where its expression is regulated by auxin	100

As a result of the previous analysis of the progeny regarding phenotypic information, the genotypes were ordered in five groups: without symptoms ("nosympt"), level 2 and 3 of root rot ("rootrot23"), level 4 and 6 of root rot ("rootrot46"), with lesion ("lesion") and the ones that died ("dead"). The analysis of the allelic frequencies in each group originated

an unrooted tree (Neighbor-Joining), that joined the closest groups (Figure 18). “lesion” and “dead” were grouped together. “nosympt” and “rootrot23”, corresponding to the most resistant, were grouped together, lastly, group “rootrot46” not linked to any other group, but closer to the “nosympt-rootrot23”.

Figure 18 – Neighbor-Joining tree of different groups of genotypes in the chestnut progeny. The groups, created regarding phenotypic information, were: “nosympt” (genotypes with no symptoms), “rootrot23” (genotypes with root rot level 2 and 3), “rootrot46” (genotypes with root rot level 4 and 6), “lesion” (genotypes with lesion) and “dead”.

Table 5 shows the alleles that appear only in the groups with no symptoms and with root rot. Frequencies of the alleles present in “nosympt” and “rootrot23” groups (corresponding to the most resistant genotypes) were summed. By analyzing Table 5, we can conclude that the allele 272 of marker Cm361 presents the highest frequency for groups “nosympt” and “rootrot23”, followed by alleles 141 and 145 of marker Cm735 and allele 177 of marker Cm433.

Table 5 – Alleles and respective frequencies obtained for groups “nosympt”, “rootrot23” and “rootrot46”: Genotypes that have the alleles are also present.

Loci	nosympt	Freq.	rootrot23	Freq.	Σ [freq (nosympt) + freq (rootrot23)]	rootrot46	Freq.	Genotypes (BDC)
Cc25	395	0,0714	395	0,17	0,2414	–	–	13, 1646, 16127

Cm3	242	0,107	242	0,17	0,277	242	0,17	1667, 1680, 1683, 1699, 16109
Cm361	272	0,5	272	0,33	0,83	272	0,33	12, 13, 1645, 1646, 1650, 1678, 1680, 1683, 1689, 1697, 1699, 16109, 16115, 16116, 16124, 16134, 16152, 16155
Cm433	177	0,0714	177	0,33	0,4014	177	0,17	1680, 1683, 1699, 16109, 16155
Cm510	281	0,0357	281	0,17	0,2057	281	0,17	1699, 16108, 16109
Cm593	325	0,0357	325	0,17	0,2057	325	0,17	1699, 16108, 16109
Cm735	141/145	0,107/0,25	141/145	0,5/0,17	0,607/0,42	141/145	0,17/0,5	12, 1645, 1667, 1669, 1680, 1683, 1690, 1691, 1699, 16100, 16103, 16105, 16108, 16109, 16124, 16134, 16155

For primer Cm361, allele 272 stands out because it was only present in the groups with no symptoms and with root rot. The combined frequency of “nosympt” plus “rootrot23” was 0,83 (Figure 19).

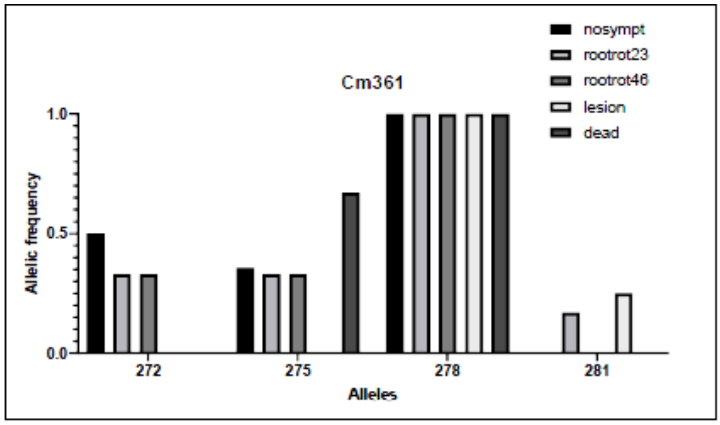


Figure 19 – Allelic frequency obtained per group for primer Cm361.

For primer Cm433, allele 177 stands out because it was only present in the groups with no symptoms and with root rot. The combined frequency of “nosympt” plus “rootrot23” was 0,4014 (Figure 20).

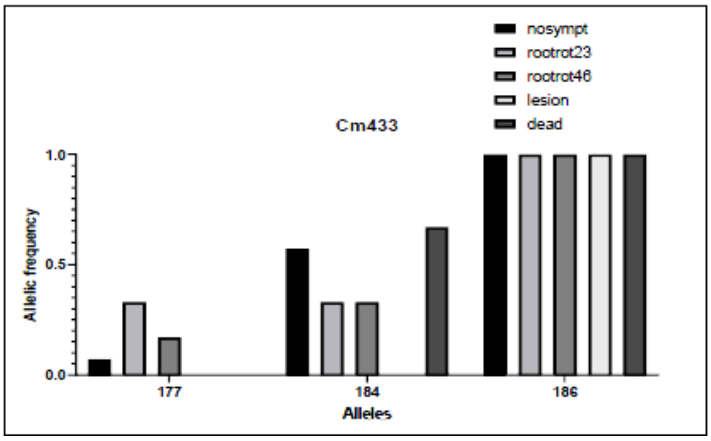


Figure 20 – Allelic frequency of each group for primer Cm433.

For primer Cm735, alleles 141 and 145 stand out because it was only present in the groups with no symptoms and with root rot. The combined frequency of “nosympt” plus “rootrot23” was 0,607 and 0,42, respectively (Figure 21).

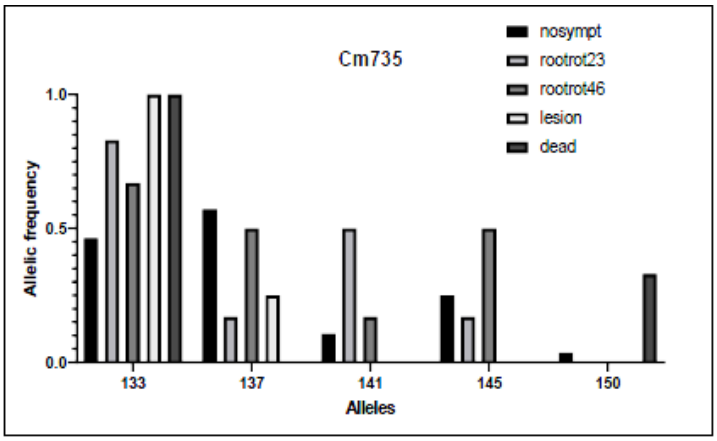


Figure 21 – Allelic frequency of each group for primer Cm735.

4. Discussion

4.1 *Micropropagation*

Clonal propagation of hybrids with improved resistance to *P. cinnamomi*, selected from the breeding program on course has been done by micropropagation with sucess. It is possible to use this method for mass propagation of selected genotype, for future release to the market. The results in the present thesis are in agreement with Navatel & Bourrain (2001) and Vahdati *et al*. (2004), the multiplication rate and rooting percentage are genotype-dependent characteristics, directly linked to the vigor of each one of the hybrids.

Chestnut is a recalcitrant species and micropropagation allows to multiply efficiently different genotypes after the optimization of culture media and hormones concentration (Santos *et al*., 2017). In recalcitrant species, rooting is the most difficult phase of the micropropagation process. The use of cytokinins in the culture media promotes juvenility (Sánchez *et al*., 1997), that favors the formation of roots. Juvenility is characterized by the high capacity for establishment *in vitro*, rapid multiplication and more easily rooting (Sánchez & Vieitez, 1991). The subculture to fresh medium every 6 to 8 weeks also allows the juvenility of plant material. Lower rooting percentage may indicate that the shoots are aged (George *et al*., 2008), meaning that they need to be subcultured in shorter intervals. The subculture between multiplication and elongation medium is also a key factor that favors rooting. Shoots that are always in the same medium tend to lose their vigor, being advisable the transfer between the media. Plants, when subjected to a hormone for a long period of time or in high concentrations, tends to have symptoms of toxicity. Hormones, namely BAP, can be toxic in high concentrations (Azizan, 2017).

Average and standard deviation were calculated for multiplication rate and rooting percentage. Through ANOVA it was confirmed that differences exist between the genotypes for multiplication rate. These differences were evaluated through Tukey test for $\alpha=0.05$, confirming that the biggest difference was observed between genotypes SM904 and SC914. For rooting percentage, no significantly different standard deviations were observed between the genotypes for $\alpha=0.05$. However, multiplication rate and rooting percentage had the same pattern (genotype SM904 with the highest value and genotype SC914 with the lowest), despite the difference between genotypes was not statistically significant for rooting. Sánchez & Vieitez (1991) calculated the multiplication rate on shoots with 6 weeks in a medium with a different composition compared with the one we used in this present study, but also with BAP. Number of shoots produced per explant varied between 1.3 and 3.1. Unlike this thesis, they discriminated between basal

sprouts and crown shoots. This study had 15 replicates, repeated at least twice, while in the present thesis 75 replicates repeated 3 times were used. We obtained multiplication rates between 1.91 and 2.54. The results of the multiplication rates obtained in both studies were similar. However, Sánchez & Vieitez (1991) obtained much lower rooting. The difference between studies may be explained by the number of cycles (with BAP medium) to which the shoots were subjected before analysis, or by the genotypes. Genotypes used in Sánchez & Vieitez (1991) were hybrids (*C. sativa* x *C. crenata*), such as the present study, with improved resistance to *P. cinnamomi*. Note also that the number of replicates and repeats were different in both studies.

In this study, it was possible to observe that SM904 has the highest multiplication rate (2.543 ± 0.617) and rooting percentage (79.6%). The genotypes were ranked with the same order in both analysis and that was SM904-SC55-SC1202-SC914, in descending order. Looking at these results, it is possible to correlate the multiplication rate with the rooting percentage. The correlation coefficient was R^2=0.8684, which means that two variables have a high association. Both multiplication and rooting are linked to plant juvenility, being the same pattern result expected. In that case, SM904 is the selected genotype with the highest vigor.

According to Santos *et al*. (2015), SC55 and SM904 are the most resistant hybrids to *P. cinnamomi*. These two hybrids had a higher multiplication rate and rooting percentage in the present work, compared with SC914, which is considered when it comes to resistance to *P. cinnamomi*. This result seems to indicate that level of resistance and vigor can also be related.

4.2 *Phenotyping*

Phenotyping the chestnut progenies allows the evaluation of their susceptibility to *P. cinnamomi*, after the inoculation of their roots with the pathogen. However, plant susceptibility is not the only variable on root rot progression, environmental conditions and pathogen virulence also plays a key role in the progress of infection (Serrazina *et al*., 2015). In the natural environment, *P. cinnamomi* is deep in the soil, which means that root rot is the first symptom of infection. The lesion of the stem is a symptom of proliferation, it represents the entry of *P. cinnamomi* into the vascular bundles. The lesion progression evaluation of root rot and external lesion of the stem, described in *Methods*, is an easy and reliable way to evaluate the susceptibility of genotypes to *P. cinnamomi.* In this study, the inoculations with the pathogen were made by infecting superficially the soil allowing the entry of *P. cinnamomi* in some wounds that may have occurred during

the inoculation in the collar zone of the plant, causing, in some cases, external lesion of the stem without root rot.

Both phenotyping and genotyping may have flaws. Phenotyping may be influenced by environmental conditions. If inoculated plants with *P. cinnamomi* are subjected to other stresses, they will become more fragile and the pathogen can take advantage of that fragility and can cause more severe damages. *P. cinnamomi* is an opportunistic pathogen that can stay in the soil for long periods until the conditions are ideal for infecting the roots of the plants. Phenotyping-genotyping association allows the discovery of alleles possibly associated with the resistance.

Comparing our results with those obtained by Santos *et al*. (2015), it is possible that 100 days is not enough time for plants developing symptoms, since we obtained 94% of survival, a much higher percentages compared with the 14% obtained by Santos *et al*. (2015), in the same 100 days essay. This may be due to the age of plants that, in the present study, were older (4 years old), while in the work cited, they were younger, 6 months old. Subsequently the 100th day after inoculation, the survived plants were kept in the same conditions. Monitorization will continue and phenotyping will be performed 1 year after inoculation again to compare with the results presented in this thesis. The high percentage of plants without symptoms may have been due to the age of the plants (older plants may need more time to show symptoms) or due to different levels of resistance. Anyway, the suggestion is to increase the inoculation time before phenotyping.

An update of the scale used by Santos *et al*. (2015) is proposed, which we think may be more rigorous for the scoring: root lesion level 1 – 0,1-5%; root lesion level 2 – 5,1-20%; root lesion level 3 – 20,1-50%; root lesion level 4 – 50,1-75%; root lesion level 5 – 75,1-100%.

4.3 *Genotyping*

DNA extraction, Qubit and NanoDrop evaluations

High quality DNA is fundamental to obtain amplification for all primers. With the optimization of the protocol for DNA extraction, a higher DNA concentration and quality were obtained when compared with the previous results obtained with the original CTAB protocol (Murray & Thompson, 1980). This optimized protocol cleans more effectively the high amount of phenols and tannins of chestnut tissues (Sanz *et al*., 2010), as a result of the increase in the number of washing steps.

Genotyping

Microsatellites were used in this work for trying to make associations between alleles with candidate genes identified for *P. cinnamomi* resistance, that codify proteins involved in different layers of defense (Freeman & Beattie, 2008; Santos *et al*., 2017). Santos *et al*. (2017) proposed a working model based on different layers of defense: physiochemical barriers, secretion of antifungal proteins and stronger cell walls, respectively, may inhibit growth and infection of *P. cinnamomi*. If *P. cinnamomi* overcomes those barriers, specific pathogen recognition may occur. Hence, host transcription is reprogramed via transcription factors and Salicylic Acid signaling. Hypersensitive Response can also be activated by many mechanisms. Cell walls may be reinforced and the secretion of antifungal proteins may increase to prevent further colonization (Santos *et al*., 2017).

Heterozygosity is positively correlated with fitness (Taylor *et al*., 2010). The number of alleles per locus and the expected and observed heterozygosity values allows the study of the genetic diversity within a population (Halima *et al*., 2012). Higher number of alleles means more genetic variation (Nei, 1987).

Polymorphism is determined by heterozygosity and number of alleles (Aljumaah *et al*., 2012), what makes microsatellites the molecular markers of choice for genetic diversity and characterization (Sheriff & Alemayehu, 2018). Polymorphism depends on the sample size, the number of detected alleles and may increase with a bigger population (Nei, 1987). Furthermore, the value of the heterozygosity is dependent on the population studied and a characteristic of each primer and it allows the selection of the most polymorphic primers. Primers with higher heterozygosity are more polymorphic, a desirable characteristic for genotyping, to better discriminate between genotypes (Powell *et al*., 1996; Jakše *et al*., 2001). Cc30 and Cm559 were the primers more informative/polymorphic, presenting the higher number of alleles and heterozygosity values, and so, the preferentially chosen for genotyping.

Previous studies suggest that primers with heterozygosity values higher than 0,5 are appropriate for genetic diversity studies (Dávila *et al*., 2009; Dorji *et al*., 2012). Genetic diversity studies performed with a higher number of microsatellite markers provide more information about the population and to compare with results from previous studies (Sheriff & Alemayehu, 2018).

The genotypes were grouped according to the phenotyping results and allelic frequencies were calculated in order to analyze genotyping data.

Neighbor-Joining tree joined the groups “nosympt” and “rootrot23”, which means that the levels 2 and 3 of root rot were more closely related to the group of plants that showed no symptoms than any other. By observation of the inoculated plants during phenotyping, we can claim that lesion of the stem is a more accurate symptom of susceptibility compared with root rot. Most of the plants that showed lesion of the stem died, and most plants that showed root rot but no stem lesion, survived. In these genotypes, the lesion was confined to the roots and didn’t spread to the stem. *C. crenata* has resistance to *P. cinnamomi*, however, by observation during phenotyping, it can display light symptoms of root rot after inoculated with *P. cinnamomi* but no progression to the stem.

The groups “lesion” and “dead” were grouped, which supports the hypothesis that lesion is more closely related to death than root rot. Level 4 and 6 of root rot is not joined with any other group but is closer to the group “nosympt-rootrot23”, showing that these three groups share more alleles that “rootrot46” with the groups “lesion” and “dead”.

Phenotyping and genotyping results were analyzed together in order to evaluate possible associations between phenotype and genotype. It is interesting to observe that alleles 141, 145, 177 and 272 are present in high frequency in the sum of groups without symptoms and with levels 2 and 3 of root rot, the most resistant. These candidate alleles related with resistance to *P. cinnamomi* were amplified with Cm primers (Cm361, Cm433 and Cm735), designed in transcriptomes of *C. mollissima* (Kubisiak *et al*., 2013). Nevertheless, QTLs were detected in the same linkage group for resistance to both pathogens by Santos *et al*. (2017) and Zhebentyayeva *et al*. (2019), which seems to indicate that both species may share similar mechanisms of resistance. These alleles together with the genotypes that have them should be further studied for the validation of these alleles as markers of resistance. Although loci amplified by the primers Cm361, Cm433 and Cm735 were present only in groups without symptoms and with root rot, making them, interesting for selection of resistant individuals, only Cm735 has known association to resistance mechanisms.

Kubisiak *et al*. (2013) proposed that only a small number of candidate genes are directly involved in the genetic basis that determines the response to the pathogen. Many others would reveal “downstream” effects that are part of the host response to the disease. Some loci are considered more important than others in this study, considering the predicted amplified gene and its predicted function and regulation. Loci that are considered putatively linked with resistance are the following: Cc3, Cc11, Cc14, Cc30, Cm3, Cm537, Cm691 and Cm735. Cc3 is related to lateral root development,

upregulated in the presence of a wound. Cc11 is related to the response to hypoxia and regulators of auxin, upregulated under stress.

The locus amplified by the primer Cc14 has a predicted amplification of *peroxisomal acyl-coenzyme A oxidase 1-like* gene, that prevents the accumulation of very-long-chain fatty acids (VLCFA) (El Hajj *et al*., 2012). It is considered to play a key role against *P. cinnamomi*. The predicted gene amplified by the primer Cc30 is *patellin-3-like*, which is involved in trafficking and regulatory roles of the plant membrane, interfering in growth and environmental stress response signaling networks (Zhou *et al*., 2019). This protein regulates auxin-mediated PIN1 relocation and plant development in *Arabidopsis thaliana* (Tejos *et al*., 2017). Loci Cc30 and Cm3 are related with plant development/stress response and defense response in the presence of biotic stress, respectively.

Locus Cm361 has the predicted expression of the gene that codes Zinc finger A20 and AN1 domain-containing stress-associated protein 8-like and naringenin,2-oxoglutarate 3-dioxygenase (F3H). The first is linked to environmental stress response, upregulated in roots and fruits during winter dormancy (temperature) (Paul & Kumar, 2015) and the second catalyzes the 3-β-hydroxylation of 2S-flavanones to 2R,3R-dihydroflavonols, which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins in plants (Han *et al*., 2017). These secondary compounds have a defense role (against herbivores and oxidative stress, for example). F3H increases under abiotic stress, like UV radiation, sucrose and ABA.

Another primer putatively related with resistance to *P. cinnamomi* is Cm537, which amplifies a gene coding for *PAT1 homolog 2-like*, which protein accumulates by being phosphorylated by MAPK4, giving rise to mRNA decay. mRNA decay via PAT1 may rapidly instigate immune responses (Roux *et al*., 2015). Upon plant pathogen recognition of a pathogen, multi-layered defense responses are activated. Transmembrane receptors can recognize conserved pathogen-associated molecular patterns (PAMPs) and the activation of MAP kinase cascades may regulate changes in gene expression, producing immune response. MAPK cascades triggers defense related pathways by transcription factors activation (Pitzschke *et al*., 2009; Tena *et al*., 2011), as proposed in a model by Santos *et al*. (2017), that describes part of the molecular interaction of *Castanea* spp. to *P. cinnamomi*'s infection.

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs) at the plant's cell surface at the time of infection (Santos *et al*., 2017). According to Coelho *et al*. (2011) *C. crenata* has about 10x more PRRs expression before inoculation compared with *C. sativa*, which may indicate that *C. crenata* has a

faster and more effective response against *P. cinnamomi*. This suggests that the earlier recognition is part of the resistance that *C. crenata* shows.
Despite all genotypes having the same response mechanisms, resistant genotypes show higher constitutive expression of candidate genes for resistance, which takes place before contact with the pathogen occurred. Resistant genotypes have, in non-inoculated conditions, secretion of antifungal proteins and stronger cell walls; these chemical and physical barriers make more difficult the infection with *P. cinnamomi* (Santos *et al*., 2017).

The primer Cm550 is considered to amplify SPX domain-containing membrane protein At4g22990-like, which is a Pi transporter in vacuolar membrane. This regulates the cytoplasmic Pi homeostasis and is required for fitness and plant growth (Liu *et al*., 2016). Primer Cm691, amplifies an auxin response factor 19-like that promotes the growth of roots and leaves, being upregulated by increasing auxin (Li *et al*., 2016). Gene *scarecrow-like* (primer Cm735), is expressed predominantly in the roots, where its expression is regulated also by auxin (Gao *et al*., 2004).

5. Conclusion and Future Perspectives

The results presented in this thesis lead us to conclude that the hybrid SM904 is the selected genotype with the highest vigor and better performance in micropropagation process. Regarding the phenotyping, the most important conclusion is that for plants aged 4 years old, the evaluation period should be longer than 100 days, the analysis should be done preferentially for more than 1 year, for more reliable scoring. An update of the scale used by Santos *et al*. (2015) is also proposed.

The alleles 141, 145, 177 and 272 were selected as having possible relevance in terms of resistance but only the 2 belonging to the primer Cm735 were considered with known association with resistance mechanisms (141 and 145). A deeper study of this topic is needed in the near future.

As a conclusion, this study allowed to obtain more knowledge of the primers used for genotyping, some of them designed by the team in *Castanea crenata* transcriptomes, and the selection of new possible resistant hybrids genotypes and interesting alleles for future Molecular Assisted Selection - MAS.

In the future, more plants need to be phenotyped and genotyped to select new resistant genotypes to propagate *in vitro*, for release to the marked, that presents a high deficit in improved plant materials. This will undoubtedly increase the knowledge on the mechanisms of resistance and allow the identification of alleles and markers associated with resistance to *P. cinnamomi*.

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7. Supplementary information

Annex 1 – CTAB Protocol for DNA extraction.

After grinding the leaf tissue, it was vortexed and placed on ice. Add in each tube 0,75 µL of 2-mercaptoethanol (1 µL/mL – 0,1% of the Extraction Buffer volume) (HOTTE) and spin at 8000 rpm for 10 minutes. Discard supernatant without disturbing the pellet, add 1 mL of OWB (Organell-Wash Buffer), vortex (mix with a white long tip if the pellet, even with vortex, keep attached to the bottom of the tube) and make another round of centrifugation (previous step) 4 times (x4) for cleaning mucilage. Re-suspend the pellet in 400 µL of OWB. Add 4 µL of RNAse (10 mg/mL) and vortex. Add 80 µL of 5% Sarkosyl and vortex for 20 seconds. Add 72 µL of 5 M NaCl. Add 80 µL of CTAB/0,7 M NaCl and 3 µL of Proteinase K (20 mg/mL) and shake by hand. Incubate at 65˚C for 12 minutes and shake several times during that period. Add 500 µL of Chloroform (HOTTE), vortex for 20 seconds and spin at 13000 rpm for 6 minutes. Pipette aqueous phase into 1.5 mL Eppendorf tubes, add 700 µL of cold 2-propanol (HOTTE) and invert several times to precipitate the DNA and keep in freezer ate -20˚C (+/- 1 hour). Spin at 13000 rpm for 10 minutes and then discard supernatant with a pipette (careful with the pellet, sometimes it's not attached to the tube). Add 1 mL of cold 70% ethanol and spin at 13000 rpm for 6 minutes and repeat (2x). Remove ethanol carefully with a pipette and clean the tube with paper towel and let it dry for 30 minutes. Add 15 µL of sterilized water in each tube and shake by hand, then transfer the content of one of the tubes to the another, to get just one tube of each genotype with 30 µL.

Annex 2 - MicroElute® DNA Clean Up Kit Centrifugation Protocol, for DNA purification.

After the sample of DNA was transferred to a clean 1.5 mL microcentrifuge tube, 60 µL of DP Buffer was added and vortexed to mix thoroughly. A MicroElute® LE DNA Column was inserted in a 2 mL Collection Tube and the mix of the previous step was transferred to the MicroElute® LE DNA Column. The column was centrifuge at maximum speed (≥13,000 x *g*) for 1 minute at room temperature and the filtrate was discarded; collection tube was reused. 700 µL of SPW Wash Buffer was added and centrifuged at maximum speed for 30 seconds; the filtrate was discarded and collection tube reused. SPW Wash Buffer wash step was repeated and the empty column was centrifuged at maximum speed for 2 minutes to dry the column. The column was transferred into a clean 1.5 mL microcentrifuge tube, 25 µL Elution Buffer was added directly to the center of column matrix and stayed at room temperature for 1 minute. The column was centrifuged at maximum speed for 1 minute and DNA was stored in the fridge at 4°C.

Annex 3 - Amplification Protocol.

Preparation of the mix with 4.75 µL of Milli-Q, 2.5 µL of 5x GoTaq® Flexi Buffer (Promega), 2 µL of dNTPs (1.25 mM), 1.25 µL of $MgCl_2$ (25 mM), 0.4 µL of M13-tailed Forward primer (2.5 µM), 0.1 µL of Reverse primer (2.5 µM), 0.4 µL of a fluorescent specific M13 sequence (2.5 nM), 0.1 µL of GoTaq® G2 Flexi DNA polymerase (5 U/µL) (Promega), 1 µL of DNA sample (5 ng/µL), to a total of 12.5 µL of PCR solution. The PCR mixtures were prepared for each primer and the PCRs are carried out using a thermocycler (Biometra).

The specific M13 sequences labeled with a fluorochrome (NED or VIC) anneal to the 5' end of each Forward.

The PCR program used was the following: 94°C for 4 minutes, 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 45 seconds, repeated 40 times. The final step is 72°C for 7 minutes.

Annex 4 - Agarose Gel Electrophoresis Protocol.

3g of Agarose D-1 Low EEO were mixed with 200 mL of 1x TBE Buffer (Invitrogen) to prepare an agarose gel and to check the presence of amplification reaction products by horizontal electrophoresis. The electrophoresis was run in 1xTBE buffer at 90 Volts for 40 minutes; the gel was visualized under UV light and the image acquisition was made with a BIO-RAD Gel-Doc equipment.

Annex 5 - Capillary Electrophoresis for fragment analysis.

Mix 0.2 µL of GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) with 12 µL of formamide to distribute to each 96 well plate. To 12.2 µL of the mix, 1 µL of PCR product was added to each well, the plate was subjected to a quick spin and the samples are denatured at 94°C for 2 minutes and further analyzed in a 8 capillary 3500 Genetic Analyzer (Applied Biosystems). The appropriate running module for the Dye Set G5 was used. 500 ROX™ Size Standard used is equivalent to LIZ 500. The duration of each run was 40 minutes.

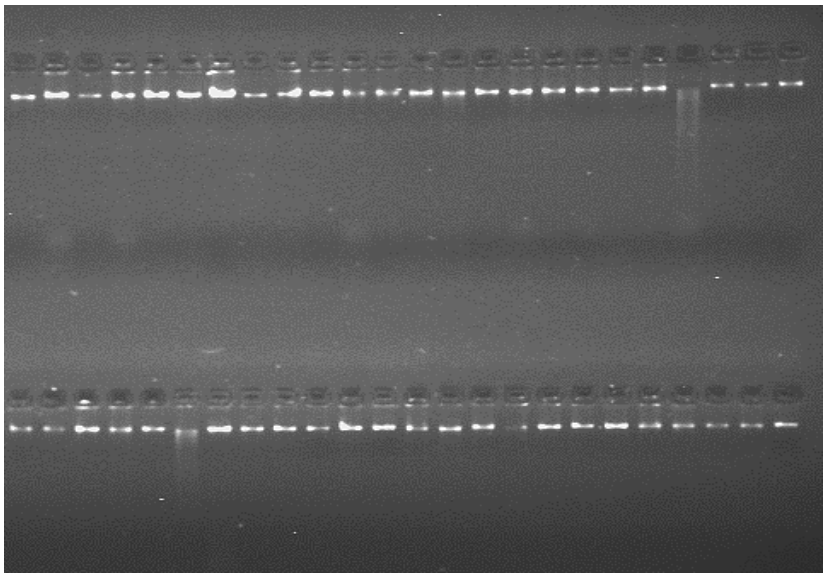
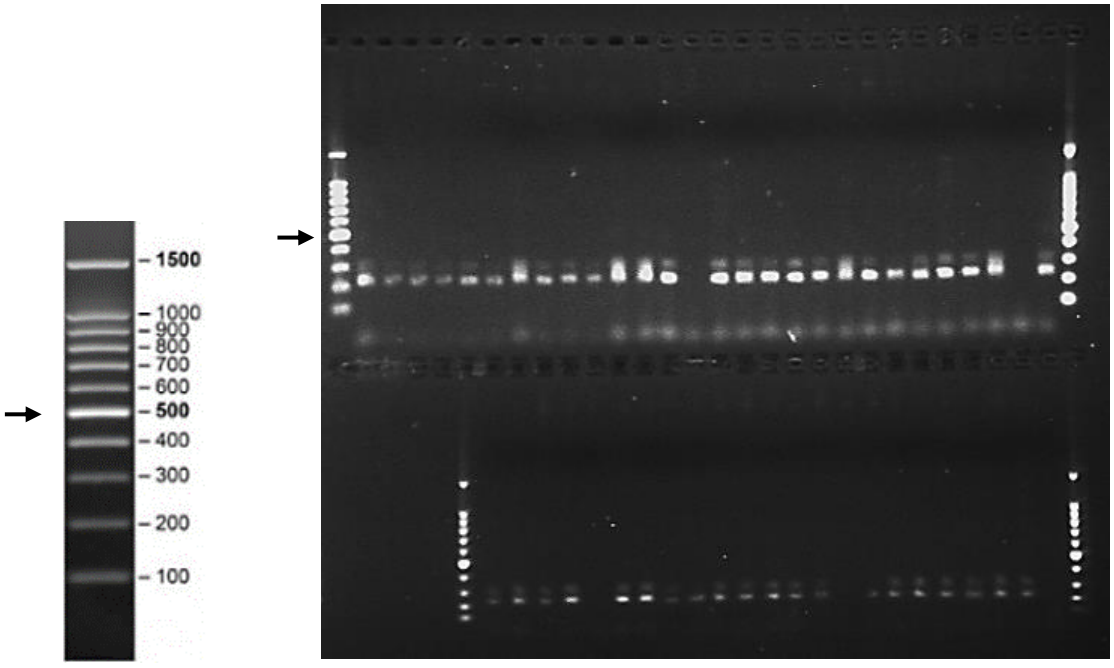
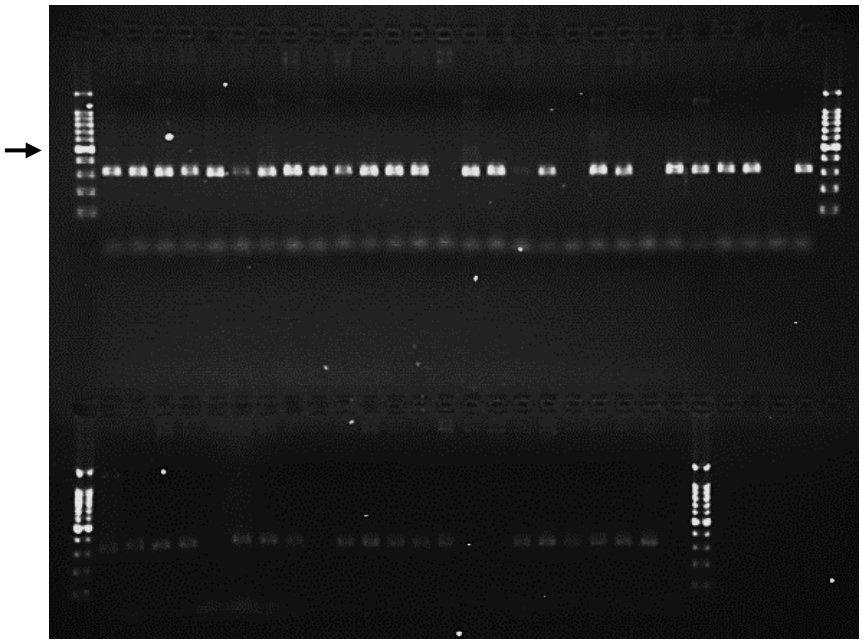


Figure S6 - DNA extraction - DNA samples (47 genotypes) in 1.5% agarose gel.



a) b)



c)

Figure S7 - PCR and Eletrophoresis – a) GRS Ladder 100 bp (grisp); b) 1.5% agarose gel of PCR products with CmSI0003 primers, with a 235 bp band (approximately); c) 1.5% agarose gel of PCR products with CmSI0593 primers, with a 315 bp band (approximately). The arrow is the three images are marking the 500 base pair band.

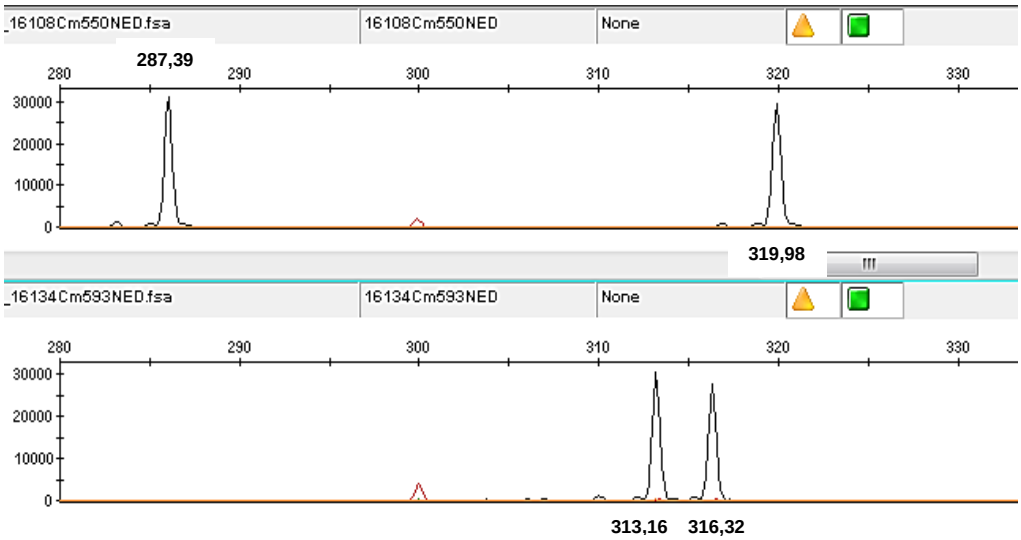


Figure S8 - Observed peaks of the individual 16108 with the primer Cm550 and the individual 16134 with the primer Cm593, respectively.

S1 Table – Medium for the maintenance and multiplication of the plantlets.

Reagents	1 L
MS Mod. No. 3B[1]	2,63 g
Sucrose	3 g
Ascorbic acid 10%	0,1 g
BAP[2] (1 g/L)	$1x10^{-4}$ g
Agar 1%	10 g

[1]Murashige & Skoog, 1962. Mod. No. 3B, by Duchefa Biochemie;

[2]Benzilaminopurine;

Add distilled water to the 1 L mark.

The pH of the medium must be 5,8 (optimal pH for shoots culture), so is needed to measure it with HI 2211 pH/ORP Meter by Hanna instruments, and regulate the pH with NaOH (strong base) or HCl (strong acid). Agar added after pH adjustment.

S2 Table – Medium for the elongation and invigoration of the plantlets.

Reagents	1 L
McCOWN WPM[3]	2,46 g
Sucrose	3 g
Ascorbic acid 10%	0,1 g
Zeatin (1 g/L)	$1x10^{-4}$ g
Agar 1%	10 g

[3]McCOWN Woody Plant Medium (Lloyd & McCown, 1980), by Duchefa Biochemie.

S3 Table – Pre-rooting medium.

Reagents	1 L
MS Mod. No. 3B[1]	2,63 g

Sucrose	3 g
Ascorbic acid 10%	0,1 g
Agar 1%	10 g
Activated charcoal (0,3%)	3 g

Adapted from Barve & Mehta, 1993.

S4 Table – V8 solution for inoculum preparation.

Reagents	1 L
Vegetables juice, type V8	200 mL
$CaCO_3$	3 g
dH_2O[4]	800 mL

[4]distilled water

S5 Table – Multiplication rates average and standard deviation per genotype.

Genotype	Average	SD
SM904	2,543	0,617
SC55	2,222	0,543
SC1202	2,029	0,463
SC914	1,908	0,328

S6 Table – Statistical analysis performed for multiplication rate: ANOVA.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	18,37	3	6,123	F (3, 296) = 11,81	P < 0,0001
Residual (within columns)	153,4	296	0,5183		
Total	171,8	299			
Data summary					
Number of treatments (columns)	4				
Number of values (total)	300				
Table Analyzed				Data 1	

ANOVA summary	
F	11,81
P value	< 0,0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0,1069

S7 Table - Statistical analysis performed for multiplication rate: Tukey test.

Number of families	1			
Number of comparisons per family	6			
Alpha	0,05			
Tukey's multiple comparisons test	**Mean Diff**	**95% CI of diff**	**Significant?**	**Summary**
SC914 vs. SC1202	-0,1389	-0,4427 to 0,1648	No	ns
SC914 vs. SC55	-0,3381	-0,6419 to -0,03437	Yes	*
SC914 vs. SM904	-0,6585	-0,9623 to -0,3548	Yes	****
SC1202 vs. SC55	-0,1992	-0,5030 to 0,1046	No	ns
SC1202 vs. SM904	-0,5196	-0,8234 to -0,2158	Yes	****
SC55 vs. SM904	-0,3204	-0,6242 to -0,01664	Yes	*

Test details	Mean 1	Mean 2	Mean Diff	SE of diff	n1	n2	q	DF
SC914 vs. SC1202	1,915	2,054	-0,1389	0,1176	75	75	1,671	296
SC914 vs. SC55	1,915	2,253	-0,3381	0,1176	75	75	4,067	296
SC914 vs. SM904	1,915	2,574	-0,6585	0,1176	75	75	7,921	296
SC1202 vs. SC55	2,054	2,253	-0,1992	0,1176	75	75	2,396	296
SC1202 vs. SM904	2,054	2,574	-0,5196	0,1176	75	75	6,25	296
SC55 vs. SM904	2,253	2,574	-0,3204	0,1176	75	75	3,854	296

S8 Table – Rooting percentages average and standard deviation per genotype.

Genotype	Rates (%)	Average	SD
SM904	79,0 78,2 74,4 87,5	79,6	4,286766
SC55	88,1 62,5 75 66,7 80	76,7	9,796888
SC1202	65,4 92,0 58,1 38,4	63,9	17,1946
SC914	58,9 77,9 48,9 61,4	61,2	9,378443

S9 Table – Statistical analysis for rooting percentages: ANOVA.

Brown-Forsythe test					
F (DFn, DFd)	1,341 (3, 13)				
P value	0,304				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	4,916				
P value	0,178				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	922,2	3	307,4	F (3, 13) = 1,651	P = 0,2263
Residual (within columns)	2421	13	186,2		
Total	3343	16			

Data summary					
Number of treatments (columns)	4				
Number of values (total)	17				

S10 Table – Phenotyping results of the symptoms in the analyzed genotypes. The genotypes that showed more symptoms are underscored at yellow and the genotypes that died due to *P. cinnamomi*'s infection are underscored at orange.

Genotype		Day of first sympthoms (0-100)	Day of death	TL (cm)	LL (cm)	RL (%)	IL (%)	RLL
BDC	12	47 (dieback)		33	0	70		6
BDC	13	47 (root rot)		50,5	0	5		2
BDC	54	33 (root rot)		26,5	2	30		5
BDC	1645			34	0	0		1
BDC	1646			29	0	0		1
BDC	1650			16	0	0		1
BDC	1667			34	0	0		1
BDC	1669	44 (dieback)		37	0	2		2
BDC	1671	35 (root rot)		25	0	70		6
BDC	1678			24	0	2		2
BDC	1680	48 (root rot)		22	0	10		2
BDC	1683			61	0	0		1
BDC	1686			42	0	0		1
BDC	1688			47	0	0		1
BDC	1689			42	0	2		2
BDC	1690			58	0	10		3
BDC	1691			43	0	0		1
BDC	1692	44 (dieback and root rot)	152	25	25	90	60	6
BDC	1697			53	0	0		1
BDC	1699			64	0	0		1
BDC	16100			24	0	2		1
BDC	16103	48 (dieback and root rot)		42	0	20		4
BDC	16104			65	0	0		1
BDC	16105	48 (dieback)		40	0	20		4
BDC	16108	48 (dieback and root rot)		28	0	10		3
BDC	16109	35 (root rot)		22	0	50		6
BDC	16111	35 (dry)	77	26	11	30	50	5
BDC	16113			61	0	2		2
BDC	16115			42	0	0		1
BDC	16116			24	0	0		1
BDC	16117			18,5	0	10		3

BDC	16118	47 (root rot)		19	0	60		6
BDC	16119			20,5	0	0		1
BDC	16123			21	0	2		2
BDC	16124			19	0	2		2
BDC	16125			27,5	0	0		1
BDC	16127	35 (root rot)		15,5	0	10		3
BDC	16128	47 (root rot)		26,5	3,5	5		2
BDC	16131			42,5	0	5		2
BDC	16134			42	0	2		2
BDC	16139			22	0	2		2
BDC	16140	47 (dieback and root rot)		36	0	5		2
BDC	16141	29 (lesion)		29	5,5	20		4
BDC	16148			18	0	5		2
BDC	16152			19,5	0	5		2
BDC	16153			32	1	10		3
BDC	16155			18	0	10		3
BDC	16156			24	0	0		1
BDC	X	48 (dry)	63	15	5	70	100	6
sativa 1		28 (external lesion and root rot)	149	31	1,5	100	10	6
sativa 2				17,5	0	50		6
crenata				34,5	0	5		2

TL	Total Length
LL	Lesion Length
RL	Root Lesion
IL	Internal Lesion
RLL	Root Lesion Level

S11 Table – Qubit and NanoDrop values for each DNA sample.

Genotype: BDC	**DNA concentration ng/µL (Qubit)**	**260/280 (Nanodrop)**	**260/230 (Nanodrop)**
12	278	1.92	2.28
13	396	1.88	2.22
54	290	1.96	2.26
1645	67	1.98	1.68
1646	206	1.7	1.17
1650	486	1.86	1.94
1667	482	1.78	1.61
1669	197	1.91	2.16
1671	312	1.94	2.20

1678	366	1.83	2.02
1680	702	1.89	2.15
1683	240	1.87	1.99
1686	105	1.91	2.01
1689	864	1.82	2.05
1690	162	1.90	2.08
1691	392	1.91	2.01
1692	362	1.90	2.18
1697	802	1.9	2.12
1699	360	1.81	1.81
16100	258	1.9	2.06
16103	39.4	1.87	1.29
16104	71.2	2.01	2.10
16105	608	1.83	1.99
16108	167	1.96	2.06
16109	366	1.91	2.21
16111	144	1.97	0.74
16113	318	1,74	1.89
16115	130	1.77	1.31
16116	216	1,74	1.69
16117	382	1.92	2.22
16118	145	1.88	2.15
16119	140	1.71	1.41
16123	684	1.89	2.10
16124	312	1.92	1.96
16125	304	1.93	2.10
16127	174	1.90	1.82
16128	181	1.88	1.93
16131	179	1.88	1.88
16134	214	1.99	2.14
16139	626	1.94	2.21
16141	40.4	1.77	1.79
16148	62.4	1.74	1.27
16152	328	1.87	2.12
16153	162	1.86	1.95
16155	328	1.91	2.15
16156	540	1.86	2.17
16162	388	1.91	2.15
X	288	1.75	1.77

S12 Table – Peak values (bp) of the alleles of the 47 genotypes and 16 primers.

A)

	Genotypes														
Primers	BDC 12	BDC 13	BDC 54	BDC 1645	BDC 1646	BDC 1650	BDC 1667	BDC 1669	BDC 1671	BDC 1678	BDC 1680	BDC 1683	BDC 1686	BDC 1689	BDC 1690
Cc 3	428.00 430.97	428.02 430.87	428.03 431.03	428.07 431.07	428.02 431.00	428.05 430.91	428.02 431.00	428.00 430.97	430.96 433.95	428.10 430.99	428.13 431.03	428.08 430.95	428.10 430.98	428.05 431.04	428.03 431.03
Cc 11	438.14	438.16	420.31 438.23	438.12	420.35 438.08	438.45	420.48 438.32	420.49 438.25	438.09	438.43	420.45 438.28	420.24 438.38	420.41 438.35	420.43 438.29	438.20
Cc 13	194.78	194.81	188.90 202.47	194.71	192.61	194.76	194.64	192.67	183.09	192.64	194.76	192.67	194.64	192.61	192.67
Cc 14	175.23 180.45	175.01 180.25	175.26 182.36	175.30 182.26	175.10 180.36	175.38 180.53	175.14 182.32	175.23 180.45	175.19 182.09	175.46 182.27	175.13 182.36	174.88 182.09	175.05 180.31	174.97 180.24	175.14 182.32
Cc 25	315.54 392.96	315.01 395.12	353.07 392.91	315.33 392.86	353.49 395.90	393.00	353.28 393.14	315.33 392.90	353.06 392.96	315.34 392.58	353.28 392.86	353.28 392.91	392.99	392.99	353.29 392.95
Cc 30	490.18 500.00	489.87 493.17	496.57 500.15	492.35 500.00	492.50 500.00	492.54 499.70	492.61 499.84	490.13 493.39	496.09 499.85	492.61 499.84	492.47 500.00	492.43 499.85	492.72 499.85	492.54 499.70	492.61 500.00
Cm 3	232.76	232.81	232.66	232.71	232.68	232.66	232.90 242.16	232.71	232.62	232.81	232.71 242.02	232.84 242.11	232.78	232.78	232.76
Cm 361	271.97 278.12	272.43 278.02	278.02	272.01 278.06	272.06 278.12	272.17 278.24	275.19 278.15	275.23 278.30	278.03	272.06 278.12	272.22 278.17	272.26 278.19	275.14 278.11	271.99 278.05	275.22 278.18
Cm 433	186.64	184.91 186.59	186.56	186.71	186.61	186.61	184.63 186.55	184.75 186.79	187.34	186.57	177.18 186.58	177.10 186.56	184.63 186.68	185.28 186.61	184.79 186.70
Cm 510	275.96 287.21	276.85 286.23	275.94 287.19	276.85 287.17	275.93 286.30	275.82 287.13	276.02 286.34	276.96 287.24	275.94	275.91 287.18	275.99 286.26	275.95 286.25	275.99 286.33	275.87 286.21	275.91 287.18
Cm 537	153.69 173.63	153.69 173.63	173.64	153.69 173.54	153.69 173.57	153.39 173.15	153.69 173.54	153.69 173.75	173.63	153.56 173.42	153.74 173.64	153.60 173.58	153.60 173.58	153.69 173.48	153.60 173.58
Cm 550	286.01 313.49	286.15 322.59	322.66	313.53	319.60 322.63	313.81	319.76	319.82 322.83	286.01 319.81	322.79	313.51 319.61	319.53	313.57 319.66	322.67	313.48 319.61
Cm 559	141.64 145.08	138.11 141.64	138.23 148.23	138.10 151.54	139.00 151.92	141.68 145.17	141.64 145.09	138.24 151.54	145.08 148.37	138.11 151.53	138.23 141.68	138.24 141.78	145.17 151.54	145.08 151.55	138.10 141.68
Cm 593	313.38 316.52	306.78 313.30	306.96 313.28	313.34 316.49	306.95 313.27	313.23 316.37	306.83 313.27	313.38 316.53	313.27	313.23 316.38	306.76 313.26	306.71 313.15	306.83 313.28	306.81 313.24	313.18 316.32
Cm 691	177.57 186.28	177.61 186.23	182.89 185.70	177.49 186.14	177.58 186.13	176.52 185.09	177.61 186.11	177.66 186.16	185.23	176.65 185.28	176.69 185.29	176.60 185.18	176.60 185.30	176.64 185.24	176.64 185.29
Cm 735	132.91 145.47	136.98	133.10 137.11	136.98 145.40	137.12	137.31 150.26	133.04 141.19	136.86 145.50	132.68	133.01	137.11 141.20	132.94 141.08	133.09	136.96	133.15 145.46

B)

	Genotypes															
Primers	BDC 1691	BDC 1692	BDC 1697	BDC 1699	BDC 16100	BDC 16103	BDC 16104	BDC 16105	BDC 16108	BDC 16109	BDC 16111	BDC 16113	BDC 16115	BDC 16116	BDC 16117	BDC 16118
Cc 3	428.08 431.05	428.09 430.96	428.06 431.07	428.12 431.01	427.97 430.98	428.93 431.67	428.03 431.03	428.10 430.99	428.09 431.08	427.95 430.94	428.69 431.43	428.13 431.02	428.03 431.03	428.19 431.07	431.04 434.04	431.03
Cc 11	438.50	438.24	438.24	420.40 438.28	438.40	420.61 438.36	438.17	420.50 438.25	420.63 438.42	438.27	420.64 438.46	438.22	438.27	438.24	438.27	438.33
Cc 13	194.90	192.79	194.76	192.73	192.73	194.78	192.64	192.64	192.76	194.69	192.64	194.74	192.55	194.69	194.78 202.58	194.78 202.56
Cc 14	175.31 182.45	175.19 182.33	175.38 182.44	175.08 182.31	175.05 182.26	175.05 182.38	174.97 180.24	175.02 180.38	175.18 182.34	175.11 182.27	175.26 180.50	175.09 182.31	174.89 180.14	174.93 180.31	175.05 181.89	174.98
Cc 25	315.45 393.00	353.15 392.88	353.17 393.00	353.48 392.83	315.45 392.97	315.45 392.97	393.00	315.29 392.94	353.43 393.03	353.29 392.84	392.88	392.82	392.84	353.39 392.90	392.77	392.68
Cc 30	492.45 499.84	490.00 499.60	490.00 493.30	490.00 493.26	492.58 499.85	489.09 492.55	492.40 499.70	492.58 499.85	486.31 490.00	489.85 500.00	492.58 500.00	492.61 500.00	489.75 493.17	499.35 526.67	489.87 496.42	489.74 496.45
Cm 3	232.78	232.76	232.66	232.78 242.10	232.74	232.78	232.78	232.77	232.82	232.73 242.08	232.90	232.80	232.84	232.76	232.82	232.73
Cm 361	275.22 278.29	275.30 278.26	272.11 278.16	272.15 278.12	275.19 278.15	275.28 278.25	275.22 278.18	275.08 278.04	275.22 278.17	272.15 278.16	275.19 278.16	275.16 278.26	271.98 278.15	272.07 278.13	278.01 281.10	277.94
Cm 433	184.87 186.79	184.86 186.78	186.61	177.16 186.55	184.63 186.68	184.74 186.68	184.62 186.68	184.56 186.61	184.57 186.62	177.31 186.71	184.87 186.80	184.13 186.58	184.92 186.61	184.86 186.56	186.42	186.49
Cm 510	275.92 287.13	276.94 287.22	275.93 286.24	276.98 281.77	276.04 286.24	276.91 287.14	275.89 287.17	275.91 287.18	277.00 281.37	275.90 281.21	275.95 285.31	276.02 287.23	275.95 287.20	275.90 287.06	278.50	275.93
Cm 537	153.26 173.21	153.69	153.56 173.54	153.60 173.76	153.40 173.33	153.43	153.74 173.61	153.69 173.54	153.69 173.75	153.69 173.63	153.39 173.24	153.74 173.64	153.69 173.54	153.69 173.57	173.60	173.66
Cm 550	313.71 319.86	313.67	313.56	313.51 319.61	319.77 322.79	313.70 319.78	319.76 322.67	319.53 322.55	319.98	319.81	287.31 322.90	313.46 319.67	313.64 319.69	322.50	319.65	319.64
Cm 559	138.24 141.78	149.29	138.11 141.64	141.68 145.17	141.68 145.17	138.62 151.83	145.17 151.54	138.11 141.63	138.11 141.64	141.64 145.08	152.18	138.23 141.68	141.68 145.03	138.23 151.53	138.23 145.08	138.10 148.35
Cm 593	313.22 316.36	313.39 316.42	306.93 313.52	313.29 325.82	306.81 313.24	313.25 316.37	313.20 316.37	313.16 316.32	313.58 325.82	313.18 325.84	307.13 313.43	313.17 316.34	313.28 316.32	313.09 316.26	313.16	312.92
Cm 691	176.57 185.16	176.68 185.28	176.73 185.21	176.64 185.24	176.69 185.21	176.69 185.23	176.69 185.29	176.64 185.17	176.69 185.16	176.69 185.28	176.60 185.23	176.65 185.22	176.73 185.28	176.73 185.39	185.29	185.29
Cm 735	137.45 145.96	133.58	137.48	133.67 141.77	137.45 145.86	137.45 145.86	137.62	133.62 145.95	133.60 141.78	137.60 141.78	133.49 150.26	137.45	137.59	133.66	133.64	133.67 137.60

C)

	Genotypes																			Parents		
Primers	BDC 16119	BDC 16123	BDC 16124	BDC 16125	BDC 16127	BDC 16128	BDC 16131	BDC 16134	BDC 16139	BDC 16141	BDC 16148	BDC 16152	BDC 16153	BDC 16155	BDC 16156	BDC X	Size range (bp)	Size average	nº alleles	Ho	SD	C1	C2
Cc 3	430.95	428.03 431.03	427.98 430.99	431.01 433.91	431.81	428.19 431.07	431.00	428.10 430.98	428.08 431.07	431.04 434.04	421.79 430.94	428.06 430.96	430.99	428.12 431.14	428.11 430.99	431.04	421,79 - 434,04	427.92	4 (421, 428, 431, 434)	41/47 = 0.872	430.96	428.08	427.92
Cc 11	438.49	420.79 438.47	420.67 438.52	438.45	437.98	450.00 490.00	438.31	438.40	438.42	438.54	438.77	420.74 438.56	438.29	438.49	438.39	438.29	420,24 - 490,00	455.12	6 (420, 427, 438, 450, 490, 540)	17/47 = 0.362	438.28	420.68 438.63	420.65 438.6
Cc 13	198.82 202.68	194.71	192.67	194.88 198.69	198.82	188.95 202.45	198.70 202.57	192.67	192.67	195.93 203.86	195.93 204.23	194.76	194.71 202.45	192.67	192.61	195.82 203.97	183,09 - 204,23	193.66	7 (183, 188, 192, 195, 198, 202, 204)	11/47 = 0.234	195.06 199.04	193.03 195.08	192.98 195.04
Cc 14	175.31 182.09	175.19 180.40	175.02 180.26	175.10	175.26	175.01	174.93	174.98 180.33	175.14 182.28	175.19	175.10	175.26 182.36	174.89 181.83	175.01 182.32	175.10 180.36	175.19 181.97	174,88 - 182,45	178.67	3 (175, 180, 182)	40/47 = 0.851	175.16	180.66 182.24	180.37 182.07
Cc 25	353.19 393.24	392.70	392.77	392.86	353.49 395.30	353.15 392.97	353.15 392.97	315.40 392.96	353.30 392.89	392.82	392.93	315.41 393.10	353.05 392.81	353.48 393.01	393.09	392.73	315,01 - 395,90	355.46	4 (315, 353, 393, 395)	31/47 = 0.66	393.13	315.06 353.34	353.69
Cc 30	499.85	490.18 500.00	489.75 493.17	499.85	430.86	490.00 496.63	499.68	492.35 500.00	490.18 500.00	490.00 499.87	489.87 496.58	492.76 500.00	499.68	489.09 492.58	492.46 499.84	489.87 499.87	486,31 - 526,67	506.49	7 (431, 486, 490, 493, 496, 500, 526)	42/47 = 0.894	489.85	492.15	489.85
Cm 3	232.94	232.84	232.65	232.63	232.96	232.76	232.76	232.87	232.74	232.84	232.82	232.59	232.72	232.78	232.74	232.68	232,66 - 242,16	237.41	2 (232, 242)	5/47 = 0.106	233.19	233.25	233.18 242.44
Cm 361	278.33	275.33 278.28	272.01 278.06	278.19 281.29	278.22	277.99	277.93	272.17 278.09	275.36 278.31	278.03	278.09	272.06 278.15	278.10 281.18	272.08 278.13	275.23 278.19	277.98	271,97 - 281,18	276.58	4 (272, 275, 278, 281)	37/47 = 0.787	278.79	272.35 275.47	272.26 275.29
Cm 433	186.90	184.74 186.66	184.87 186.56	186.58	186.68	186.51	186.58	184.92 186.49	184.79 186.71	186.73	186.68	186.55	186.51	177.13 186.51	184.61 186.67	186.54	177,10 - 187,34	182.22	3 (177, 184, 186)	27/47 = 0.574	186.97	184.43	177.38 184.96
Cm 510	275.95	276.02 286.29	276.88 287.19	275.93	277.17	275.99	275.92	275.95 287.21	276.04 287.25	275.88	276.99	276.02 286.28	275.95 288.51	276.94 286.33	276.03 287.18	275.98	275,82 - 288,51	282.17	5 (276, 278, 281, 286, 288)	36/47 = 0.766	281.12 286.04	286.29	281.41 286.41
Cm 537	173.31 175.39	153.81 173.60	153.69 173.66	169.59 173.61	173.23 175.31	173.70	173.48 175.55	153.82 173.54	153.69 173.51	173.51	173.56	153.69 173.48	173.66	153.60 173.74	153.69 173.48	173.60 175.64	169,59 - 175,64	172.62	4 (153, 169, 173, 175)	36/47 = 0.766	173.7	153.85	152.83
Cm 550	287.27 317.14	286.97 314.11	322.54	316.70 319.50	319.93	322.62	286.88	313.56 319.64	319.79 322.79	319.62	286.03 319.54	313.75 319.86	287.50	313.81	319.69 322.71	316.72 319.62	286,03 - 322,90	304.47	5 (286, 313, 317, 319, 322)	26/47 = 0.553	313.95 320.19	313.84 323.01	313.89 320.03
Cm 559	145.86 157.92	138.48 142.09	145.08 151.54	138.10	138.74 145.72	138.10 141.54	138.10 148.21	138.24 151.53	141.64 145.08	138.87 145.87	138.48 145.43	138.10 151.56	139.00 158.33	141.63 145.08	138.10 151.54	139.00 157.26	138,10 - 157,39	147.75	6 (138, 142, 145, 148, 151, 158)	44/47 = 0.936	missing value	142.19 152.04	142.28 148.75
Cm 593	313.33	313.23 316.38	313.32 316.48	313.26	313.23	313.09	313.04	313.16 316.32	313.23 316.38	313.23	313.30	306.74 313.10	312.92	306.86 313.33	313.16 316.32	313.15	306,71 - 325,84	316.28	4 (306, 313,316,325)	35/47 = 0.745	313.53	307.01 316.47	307.1 326.07
Cm 691	182.51 185.29	176.68 185.28	176.61 185.23	182.45 185.22	183.04 185.95	182.35 185.23	185.34	176.64 185.29	176.73 185.28	182.39 185.28	182.33 185.23	176.60 185.17	185.23	176.56 185.23	176.64 185.24	182.39 185.29	173,79 - 186,16	179.98	3 (177, 182, 186)	42/47 = 0.894	186.08	177.5	177.46
Cm 735	137.46	133.58	133.58 146.00	133.56 137.44	133.06	133.60	133.60	137.58 146.00	137.58	133.60	133.70	137.57	133.04	133.57 141.72	133.70	133.61	132,68 - 146,00	139.34	5 (133, 137, 141, 145, 150)	22/47 = 0.468	133.44 137.44	146 150.26	141.57 150.39

S13 Table – Allelic frequencies calculated for each primer and group, used in PopTree software. “nosympt” = genotypes without symptoms; “rootrot23” = genotypes with levels 2 and 3 of root rot; “rootrot46” = genotypes with levels 4 and 6 of root rot; “lesion” = genotypes with lesion; “dead” = genotypes that died.

5 population
nosympt
rootrot23
rootrot46
lesion
dead

locus 1	**Cc3**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc3	421	*	0,036	0	0	0	0
	428	*	0,857	0,67	0,67	0,5	0,67
	431	*	1	1	1	1	1
	434	*	0,036	0,17	0,143	0,25	0
locus 2	**Cc11**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc11	420	*	0,357	0,33	0,33	0,25	0,33
	438	*	1	1	1	0,75	1
	490	*	0	0	0	0,25	0
	540	*	0	0	0	0,25	0
locus 3	**Cc13**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc13	183	*	0	0	0,17	0	0
	188	*	0	0	0	0,5	0
	192	*	0,464	0,5	0,17	0	0,67
	195	*	0,464	0,33	0,67	0,5	0,33
	198	*	0,107	0,17	0	0	0
	202	*	0,0714	0,17	0,17	0,75	0
	204	*	0,0357	0	0	0,25	0,33
locus 4	**Cc14**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc14	175	*	1	1	1	1	1
	180	*	0,464	0	0,33	0	0,33
	182	*	0,429	0,83	0,5	0,5	0,67
locus 5	**Cc25**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc25	315	*	0,32	0	0,5	0	0
	353	*	0,286	0,83	0,33	0,75	0,33
	393	*	0,93	0,83	1	1	1
	395	*	0,0714	0,17	0	0	0

locus 6	**Cc30**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc30	430	*	0	0,17	0	0	0
	486	*	0	0,17	0	0	0
	490	*	0,32	0,5	0,67	0,5	0,67
	493	*	0,75	0,5	0,33	0	0,33
	496	*	0,36	0,17	0,33	0,5	0
	500	*	0,75	0,33	0,67	0,75	1
	526	*	0,036	0	0	0	0

locus 7	**Cm3**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm3	232	*	1	1	1	1	1
	242	*	0,107	0,17	0,17	0	0

locus 8	**Cm361**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm361	272	*	0,5	0,33	0,33	0	0
	275	*	0,357	0,33	0,33	0	0,67
	278	*	1	1	1	1	1
	281	*	0	0,17	0	0,25	0

locus 9	**Cm433**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm433	177	*	0,0714	0,33	0,17	0	0
	184	*	0,571	0,33	0,33	0	0,67
	186	*	1	1	1	1	1

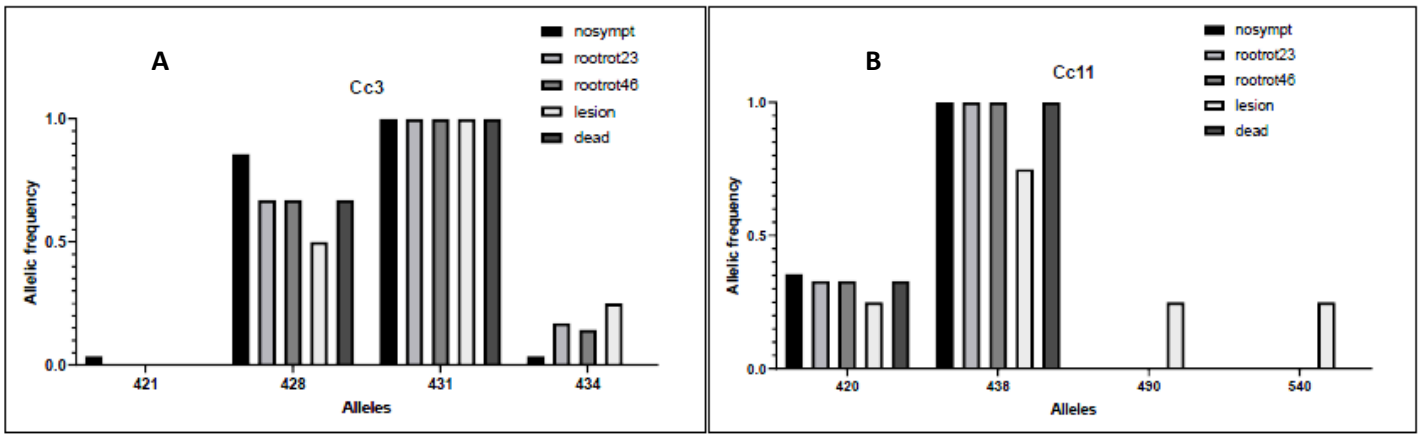
locus 10	**Cm510**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm510	276	*	1	0,83	1	1	1
	278	*	0	0,17	0	0	0
	281	*	0,0357	0,17	0,17	0	0
	286	*	0,82	0,5	0,5	0,25	0,67
	288	*	0	0	0	0,25	0

locus 11	**Cm537**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm537	153	*	0,857	0,67	0,67	0	0,67
	169	*	0,0357	0	0	0	0
	173	*	1	1	0,83	1	0,67
	175	*	0,0714	0,17	0	0	0,33

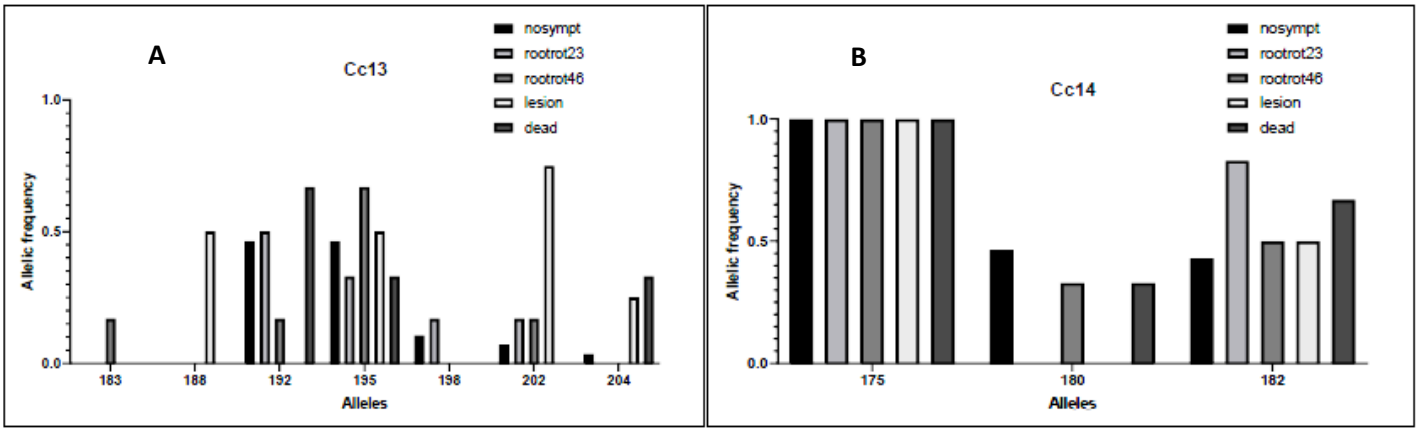
locus 12	**Cm550**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cc550	286	*	0,179	0	0,33	0,25	0,33
	313	*	0,321	0,5	0,33	0	0,33
	317	*	0,0714	0	0	0	0,33
	319	*	0,607	0,83	0,83	0,25	0,33
	322	*	0,393	0	0,17	0,5	0,33

locus 13	**Cm559**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**

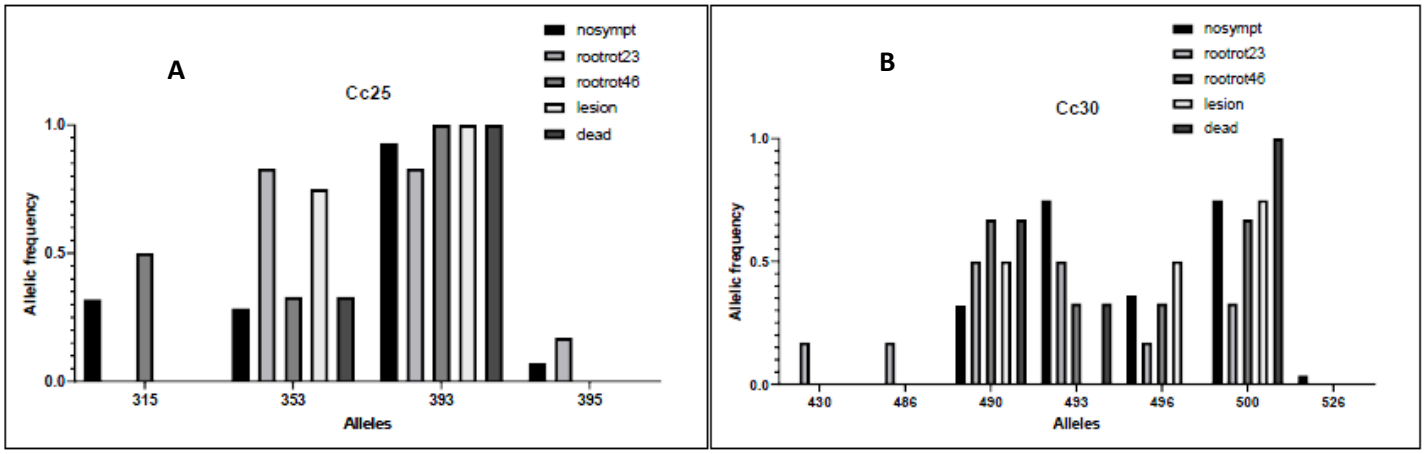
Cc559	138	*	0,607	0,83	0,5	1	0,33
	141	*	0,429	0,67	0,5	0,25	0
	145	*	0,429	0,5	0,5	0,25	0
	148	*	0,0357	0	0,33	0,25	0,33
	151	*	0,429	0	0,17	0	0,33
	158	*	0,0357	0	0	0,25	0,33
locus 14	**Cm593**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm593	306	*	0,321	0,33	0	0,25	0,33
	313	*	1	1	1	1	1
	316	*	0,5	0,17	0,5	0	0,33
	325	*	0,0357	0,17	0,17	0	0
locus 15	**Cm691**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm691	177	*	0,857	0,67	0,67	0	0,67
	182	*	0,107	0,17	0	0,75	0,33
	186	*	1	1	1	1	1
locus 16	**Cm735**		**nosympt**	**rootrot23**	**rootrot46**	**lesion**	**dead**
Cm735	133	*	0,464	0,83	0,67	1	1
	137	*	0,571	0,17	0,5	0,25	0
	141	*	0,107	0,5	0,17	0	0
	145	*	0,25	0,17	0,5	0	0
	150	*	0,0357	0	0	0	0,33



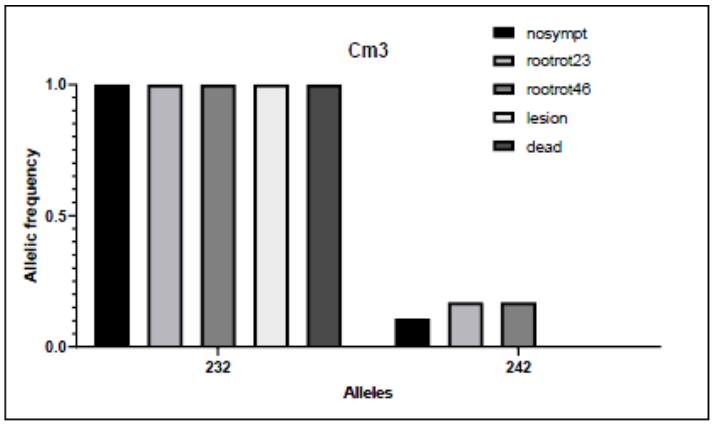
S14 Figure - Allelic frequency of each group for primers Cc3 (A) and Cc11 (B).



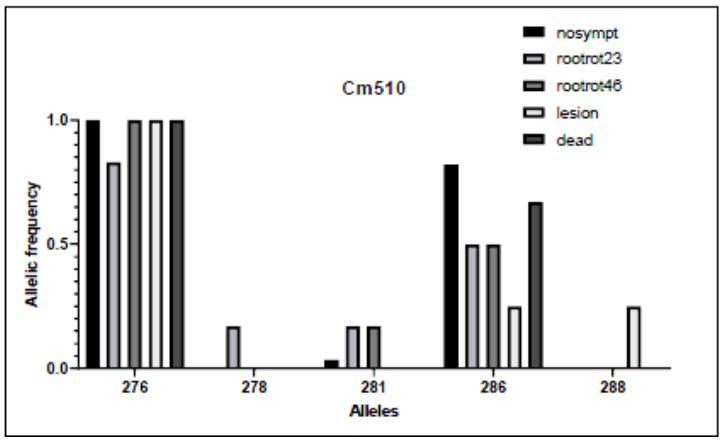
S15 Figure - Allelic frequency of each group for primers Cc13 (A) and Cc14 (B).



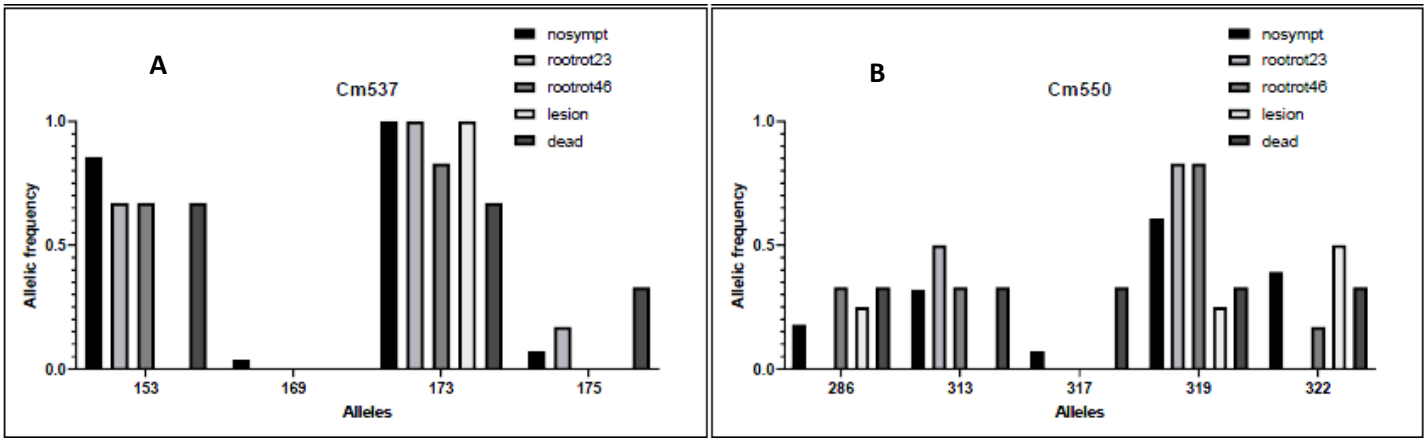
S16 Figure - Allelic frequency of each group for primers Cc25 (A) and Cc30 (B).



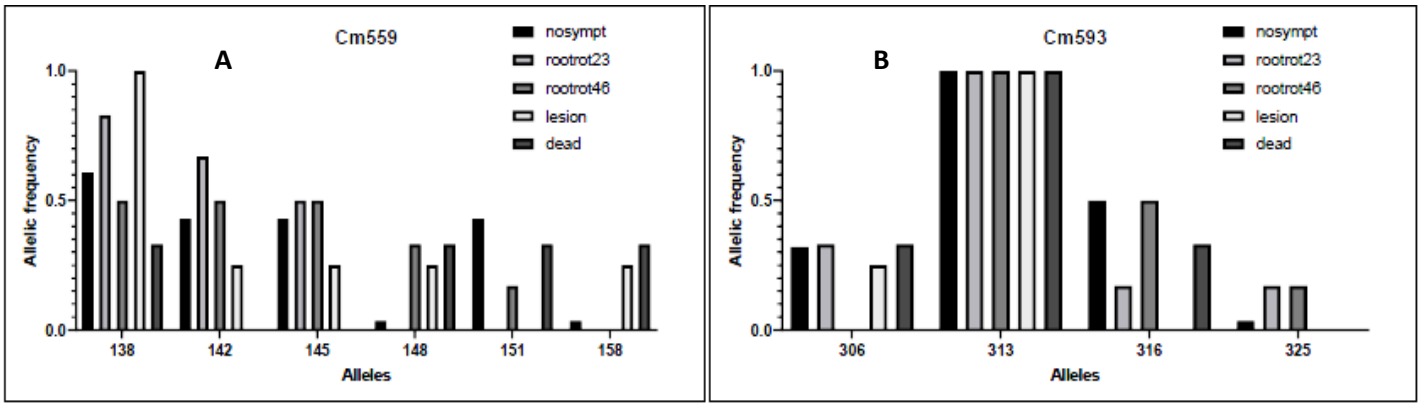
S17 Figure - Figure 20 – Allelic frequency of each group for primers Cm3.



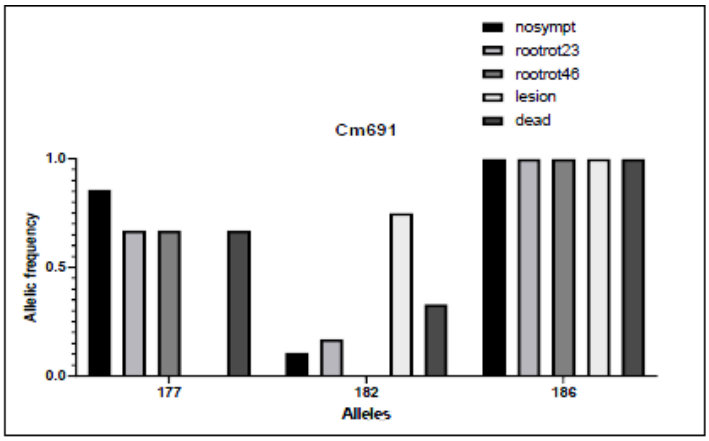
S18 Figure - Allelic frequency of each group for primer Cm510.



S19 Figure - Allelic frequency of each group for primers Cm537 (A) and Cm550 (B).



S20 Figure - Allelic frequency of each group for primers Cm559 (A) and Cm593 (B).



S21 Figure - Allelic frequency of each group for primer Cm691.